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EVALUATION OF ALTERATIONS IN DNA METHYLATION ASSOCIATED WITH THE 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD)-INDUCED INHIBITION OF DIFFERENTIATION IN LIPOPOLYSACCHARIDE (LPS)-STIMULATED MURINE SPLENOCYTES

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Emily Ann McClure

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# EVALUATION OF ALTERATIONS IN DNA METHYLATION ASSOCIATED WITH THE 2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)-INDUCED INHIBITION OF DIFFERENTIATION IN LIPOPOLYSACCHARIDE (LPS)-STIMULATED MURINE SPLENOCYTES

By

Emily Ann McClure

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ABSTRACT

EVALUATION OF ALTERATIONS IN DNA METHYLATION ASSOCIATED WITH THE 2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)-INDUCED INHIBITION OF DIFFERENTIATION IN LIPOPOLYSACCHARIDE (LPS)-STIMULATED MURINE SPLENOCYTES

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Splenic B-cells isolated from mice injected with LPS, differentiate into antibody-producing plasma cells *in vitro*. Pretreatment with TCDD, a potent immunosuppressive agent, impairs B-cell differentiation. Altered DNA methylation, an epigenetic event, may play a key role in this impairment. DNA was isolated from splenocytes prepared 6 days post experiment initiation from 5-6wk old female C57BL/6 mice dosed with: TCDD, 3 or 30μg/kg, on day 0; or LPS, 25μg/mouse, on day 4; or sequentially with TCDD and then LPS on days 0 and 4, respectively. To discern regions of altered DNA methylation (RAMs), DNA was restricted with HpaII (a methylation sensitive enzyme), followed by arbitrarily primed PCR and capillary electrophoresis. The mRNA expression of selected genes (annotated RAMs or genes closely affected by them) that might affect B-cell differentiation were analyzed using qRT-PCR. LPS, 3, or 30μg/kg TCDD alone resulted in 40, 43 and 42 RAMs, respectively, while LPS challenge subsequent to 3 or 30μg/kg TCDD resulted in 34 and 39 RAMs, respectively. Interestingly, the combined treatments lead to many RAMs observed in single treatments but also many unique RAMs. Three patterns of mRNA expression were observed: no change, similar changes in all groups, and different changes based upon treatment. Collectively, this research suggests a novel epigenetic mechanism potentially regulating gene expression stimulated by LPS and TCDD exposure and important in the differentiation of B-cells in the splenocyte population.

I dedicate this work to all those who labor to the benefit of others.

There is no psychiatrist in the world like a puppy licking your face

-Ben Williams

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## A

**Adcy5** Adenylate cyclase type 5.

**AFCs** Antigen Forming Colonies.

**Ag** antigen.

**AhR** aryl-hydrocarbon receptor.

**AIP** aryl-hydrocarbon receptor interacting protein.

**Akt** protein kinase B.

**Aldh3a1** aldehyde dehydrogenase 3 family, member A1.

**AP-1** activator protein 1.

**AP-PCR** Arbitrarily Primed PCR.

**ARE** aryl-hydrocarbon response element.

**ARNT** aryl-hydrocarbon nuclear translocator.

**ASC** antibody-secreting cell.

## B

**BACH2** BTB and CNC homology 2.

**BAFF** B-cell activating factor.

**BAFFR** B-cell activating factor (BAFF) receptor.

**Bank1** B-cell scaffold protein with ankyrin repeats.

**Bcl-xl** B-cell lymphoma-extra large.

**Bcl6** B-cell lymphoma 6 protein.

**Bcor** Bcl6 co-repressor.

**BCR** B-cell receptor.

**Bim** Bcl2-like protein 11.

**BLC** B-lymphocyte chemoattractant.

**Blimp1** B-lymphocyte-induced maturation protein 1.

**BLNK** B-cell linker protein.

**Btk** Bruton's tyrosine kinase.

C

$Ca^{2+}$ calcium.

**Cadps2** Calcium-dependent secretion activator 2.

**CaM** calmodulin.

**CCL1** chemokine (C-C motif) ligand 1.

**CD14** cluster of differentiation 14.

**CE** Capillary Electrophoresis.

**CREB** cAMP response element-binding protein.

**Cyp1** cytochrome P450, family 1.

**Cyp1a1** cytochrome P450, family 1, subfamily A, polypeptide 1.

D

**DAG** diacylglycerol.

**DAVID** Database for Annotation, Visualization and Integrated Discovery.

**DD** death domain.

**Ddx54** Dead box 54.

**DNMT** DNA methyltransferase.

**DRE** dioxin response element.

E

**EBF** early B-cell factor.

**Ebf1** transcription factor COE1.

**ER** endoplasmic reticulum.

**EREG** epiregulin.

**ERK** extracellular signal-regulated kinase.

**EsR** estrogen receptor.

**Ets1** v-ets erythroblastosis virus E26 oncogene homolog 1.

**F**

**Fli1** friend leukemia virus integration 1.

**FoxO** forkhead box protein O.

**G**

**GO** Gene Ontology.

**GPCR** G-Protein Coupled Receptor.

**GSK-3β** glycogen synthase kinase 3β.

**Gsta1** glutathione S-transferase, alpha 1 (Ya).

**H**

**HDAC** histone deacetylase.

**Hic-1** hypermethylated in cancer-1.

**Hrs** hepatocyte growth factor-regulated tyrosine kinase substrate.

**Hsp90** heat shock protein 90kDa.

**I**

**IκB** inhibitor of nuclear factor of κ light chain gene enhancer in B-cells.

**IgJ** immunoglobulin J.

**IgM** immunoglobulin M.

**IKK** inhibitor of nuclear factor of κ light chain gene enhancer in B-cells (IκB) kinase.

**IL-1** interleukin-1.

**IL-8** interleukin-8.

**Il17rd** Interleukin-17 receptor D.

**ILL** innate-like lymphocyte.

**$IP_3$** inositol triphosphate.

**$IP_3R$** inositol triphosphate receptor.

**IRAK** interleukin-1 (IL-1) receptor associated kinase.

**IRF3** interferon regulatory factor 3.

**IRF4** interferon regulatory factor 4.

**J**

**JNK** c-Jun N-terminal kinase.

**K**

**Krr1** small subunit processome component homolog.

**L**

**LBP** lipopolysaccharide (LPS) binding protein.

**LINE** long interspersed nuclear element.

**LPS** lipopolysaccharide.

**Lyn** V-yes-1 Yamaguchi sarcoma viral related oncogene homolog.

**M**

**mϕ** macrophages.

**MAPK** mitogen-activated protein kinase.

**MD1** lymphocyte antigen 86.

**MD2** lymphocyte antigen 96.

**Mdm2** murine double minute 2 oncogene.

**MHC** major histocompatability complex.

**MITF** microphthalmia-associated transcription factor.

**mTOR** mammalian target of rapamycin.

**MyD88** Myeloid differentiation primary response gene 88.

**MZ** marginal zone.

**N**

**NFκB** nuclear factor κ-light-chain-enhancer of activated B-cells.

**NK** natural killer cell.

**Nrf2** NF-E2 related factor 2.

**P**

**p23** heat shock protein co-chaperone p23/prostaglandin E synthase 3.

**p27**$^{kip1}$ cyclin-dependent kinase inhibitor 1B.

**p300** E1A binding protein p300.

**p38** p38 mitogen-activated protein kinase (MAPK).

**p53** tumor protein 53.

**PAMPs** pathogen-associated molecular patterns.

**Pax5** paired box protein 5.

**PBS** phosphate buffered saline.

**PDK** pyruvate dehydrogenase kinase.

**PDK1** 3-phosphoinositide dependent protein kinase-1.

**Phlpp** PH domain and leucine rich repeat protein phosphatase.

**$PI_3K$** phosphoinositide 3-kinase.

**$PIP_2$** phosphatidylinositol (4,5)-bisphosphate.

**$PIP_3$** phosphatidylinositol (3,4,5)-triphosphate.

**PKC** protein kinase C.

**PLC** phospholipase C.

**PLCγ** phospholipase C γ.

**PRRs** pattern recognition receptors.

**PTEN** phosphatase and tensin homolog.

**Ptpn3** Tyrosine-protein phosphatase non-receptor type 3.

**PU.1** transcription factor PU.1.

## Q

**qRT-PCR** quantitative reverse transcription PCR.

## R

**RAG** recombination activating gene.

**Ralgds** Ral guanine nucleotide dissociation stimulator.

**RAM** region of altered DNA methylation.

**RAMs** regions of altered DNA methylation.

**Rarα** retinoic acid receptor α.

**RB** retinoblastoma protein.

**REN** renin.

**RIP** receptor-interacting serine-threonine kinase.

**RP105** lymphocyte antigen 64, CD180 molecule.

**S**

**SAM** S-adenosyl methionine.

**SpiB** Spi-B transcription factor.

**SRBC** sheep red blood cell.

**STAT5** signal transducer and activator of transcription 5.

**Syk** spleen tyrosine kinase.

**T**

**TAK1** transforming growth factor β (TGFβ) activated kinase 1.

**TBK1** TANK-binding Kinase 1.

**TBP** TATA-binding protein.

**TCDD** 2,3,7,8-tetrachlorodibenzo-ρ-dioxin.

**Tcf** T-cell factor/transcription factor.

**TGFβ** transforming growth factor β.

**TI** T-cell independent.

**TIR** toll/interleukin-1 receptor.

**TIRAP** toll/interleukin-1 receptor (TIR) domain containing adaptor protein.

**TLR4** toll-like receptor 4.

**TLRs** toll-like receptors.

**TNF** tumor necrosis factor.

**TRAF6** tumor necrosis factor (TNF) receptor associated factor 6.

**TRAM** TRIF-related adaptor molecule.

**TRIF** TIR domain-containing adaptor-inducing interferon-β.

**TSC2** tuberous sclerosis complex 2.

**TSS** transcriptional start site.

**U**

**Ube2l6** Ubiquitin/ISG15-conjugating enzyme E2 L6.

**Ube3A** ubiquitin protein ligase E3A.

**W**

**WT** wild type.

**X**

**Xbp1** X-box binding protein 1.

**Z**

**Zfp128** zinc finger protein 128.

# 1. INTRODUCTION

Foreign antigen protection in vertebrates is directed by a multicomponent immune system. Immune responses are divided into two main categories: innate and adaptive. These two immune responses work synergistically and in tandem to protect the animal from foreign pathogens and toxicants, e.g. bacteria, viral capsids, and venoms. Attenuation of immune response(s) results in increased incidence of illness and death in response to infection. Hypersensitivity results in varied responses ranging from inflammation and allergic reaction to (when allowed to continue out of control) death.

The adaptive immune system is composed of T-cells and B-cells specialized to eliminate and neutralize antigens as well as to maintain memory of previous exposure. The response is highly specific, with each mature T- or B-cell recognizing only one antigen. Successful antigen recognition results in rapid activation and proliferation of stimulated lymphocytes. It is this response that is targeted in vaccinations and efficiently clears foreign antigens from the body due to the specificity of the response. However, an adaptive immune response is not fully triggered until $\geq$96hrs after initial infection. The one exception to this rule is allergic reaction, which is rapidly triggered by memory cells in response to antigen exposure after an initial sensitizing exposure. As the animals used in this study were kept in clean rooms and exposed to neither LPS nor 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) prior to experimental administration, it is unlikely that any of the reactions observed will be a result of allergic reaction. Immune responses occurring before the adaptive response belong to the innate immune response.

The innate immune response mounts the first response to foreign antigens. These first responses occur within 4hrs of exposure and are mediated by natural killer cells (NKs), dendritic cells, macrophagess (mϕs), neutrophils, and innate-like lymphocytes (ILLs). LPS

in the outer membrane of gram-negative bacteria, bacterial flagella, unmethylated CpG sequences in bacterial DNA, and viral double-stranded RNA contain repetitive structures known as pathogen-associated molecular patterns (PAMPs) which are recognized by cells of the innate immune system through pattern recognition receptors (PRRs).

Many of the the cells involved in innate immunity mature and are initially activated within the spleen. The spleen filters and recognizes foreign antigens from the blood while also serving as a repository for monocytes and a location for the maturation of B- and T-cells. In fact, the average spleen is composed of 45-50% B-cells (Crawford and Kaminski, unpublished data). Many of the pathways commonly hypothesized to occur in B-cells are not actually observed nor described in B-cell populations, although they are likely relevant as they have been observed in multiple cell lineages (mϕs, dendritic cells, and cultured cell lines) (Peng, 2005). For this reason, and the high concentration of B-cells regularly isolated from the spleen, this research will focus primarily on B-cells and the potential interactions occurring therein as a response to LPS challenge and TCDD exposure.

Triggering of many signaling pathways is critical to mounting appropriate innate and adaptive immune responses. Inappropriate signaling through altered regulation of lineage, development, or differentiation-specific genes (especially transcription factors) leads to inappropriate immune responses varying from senescence to lymphoma.

TCDD exposure prior to LPS stimulation causes animals and immune cell lines to fail in mounting appropriate response(s) (Agency for Toxic Substances and Disease Registry (ATSDR), 1998). TCDD's efficient inhibition of innate (and adaptive) immunity can be used to assess mechanisms necessary for the differentiation of immunologic cells.

Current knowledge indicates that TCDD-induced suppression of the primary humoral immune response occurs upstream of antibody (immunoglobulin M (IgM)) production and B-lymphocyte-induced maturation protein 1 (Blimp1) upregulation (North et al., 2009).

Where in the upstream signaling pathways TCDD inhibits signaling for future Blimp1 expression and whether this inhibition is directed by genetic or epigenetic mechnanisms is not yet understood.

Embryonic exposure to TCDD increases DNA methylation and decreases expression of imprinted H19 and Igf2 genes (Wu et al., 2004) while also inducing cytochrome P450, family 1, subfamily A, polypeptide 1 (Cyp1a1). These changes in DNA methylation and gene expression are accompanied by an increase in methyl transferase activity (Wu et al., 2004). This research will further these results by investigating changes in DNA methylation resulting from LPS and TCDD exposure and evaluating whether concomitant exposure alters the methylation patterns induced by either treatment in isolation.

## 1.1 B-cell Differentiation and Maturation

B-cells are members of the immune system whose different subsets perform various roles associated with innate immunity (within 4hrs of exposure), the early induced innate response (4-96hrs of exposure), and the adaptive immune response ($\geq$96hrs after exposure). B-cells begin development as hematopoeitic stem cells in the bone marrow and differentiate into multipotent progenitor cells. Multipotent progenitor cells differentiate into early lymphoid progenitor cells, which further differentiate into common lymphoid progenitors, the progenitor cell population from which B-cells, T-cells, and NKs derive. B-cells are further subdivided into two groups: B1 and B2 B-cells.

B1 B-cells comprise $\sim$5% of all B-cells in mice and appear first during fetal development. In adult mice, B1 B-cells are found in the spleen and intestine, as well as the peritoneal and pleural cavities (Kantor and Herzenberg, 1993). Because they represent

such a small portion of B-cells and because they are mostly found in peritoneal and pleural cavities, the development of B1 B-cells is beyond the scope of this research.

Non self-reactive, surface IgM-expressing B-cells (B2 B-cells) generated in the bone marrow migrate to the spleen and undergo further maturation into marginal zone (MZ) and follicular B-cells (Pillai and Cariappa, 2009; Dorshkind and Montecino-Rodriguez, 2007). In secondary lymphoid tissues, differentiation is divided into 3 phases: pre-germinal center, germinal center, and post-germinal center stages. MZ B-cells do not recirculate like B1 B-cells, rather they react to pathogens trapped by mϕs in the spleen MZ and likely are uniquely adapted to provide the first response to pathogens reaching the bloodstream. These resting mature B-cells express low CD23, high MHCI, CD35, and CD21 and do not require T-cell help for activation. In rodents, $IgM^{+}CD27^{+}$ MZ B-cells are restricted to the spleen (Pillai and Cariappa, 2009).

During development, immunoglobulin rearrangement begins in pro-B-cells in the bone marrow and regulates the formation of plasma cells and memory cells. IL-7 signaling promotes E2A expression, which cooperates with transcription factor PU.1 (PU.1) to induce early B-cell factor (EBF) expression. E2A and EBF work together to drive expression of pro-B proteins such as recombination activating gene (RAG)1, RAG2, and paired box protein 5 (Pax5) (Gupta et al., 2007). Pax5 induces B-cell linker protein (BLNK) expression (Gupta et al., 2007) (see section 1.1.5 for BLNK signaling). Transcription factors Pax5, B-cell lymphoma 6 protein (Bcl6), microphthalmia-associated transcription factor (MITF), v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1), and BTB and CNC homology 2 (BACH2) repress antibody-secreting cell (ASC) differentiation (Shapiro-Shelef and Calame, 2005).

RAG genes induce V(D)J rearrangement in B- and T-cells and are expressed at two times during B-cell differentiation in order to rearrange the heavy and light chains of the

B-cell receptor (BCR). VpreB1 and λ5 gene protein products associate to form a surrogate light chain which chaperones newly synthesized $\mu$ chains to the cell surface in the form of the pre-BCR (Szutorisz et al., 2005). Signaling from this receptor then mediates signaling leading to proliferation of pre-B-cells with productive heavy chain rearrangement. Immature B-cells expressing rearranged BCR are tested for self-reactivity in bone marrow before localization to the spleen to complete differentiation.

Naïve B-cells with appropriate in-frame immunoglobulin rearrangements travel to and mature in the spleen (Cariappa and Pillai, 2002). $IgM^{+}IgD^{-}$ immature B-cells in bone marrow acquire the ability to emigrate to spleen red pulp and become $IgM^{hi}IgD^{lo}CD21^{lo}CD23^{-}$ newly formed B-cells (Cariappa and Pillai, 2002). At high concentrations, T-cell independent (TI)-1 antigens (e.g. LPS) do not require T-cell help to induce polyclonal activation of B-cells. TI-2 antigens (capsular polysaccharides) act by cross-linking the BCR, although excessive crosslinking causes B-cells to become anergic. Dendritic cells and macrophages provide co-stimulatory molecule BAFF which is recognized by the BAFF receptor (BAFFR) and plays an important role in directing follicular B-cell survival.

Immature B-cells remain for ~1 day in spleen red pulp, then colonize spleen lymphoid follicles where they express high levels of IgD and CD23 (Cariappa and Pillai, 2002). A proportion of follicular B-cells express intermediate levels of CD21 and acquire the ability to recirculate, giving rise to $IgD^{hi}IgM^{lo}CD23^{+}CD21^{int}$ mature recirculating naïve follicular B-cells (Cariappa and Pillai, 2002). A subpopulation of $IgM^{hi}IgD^{hi}$ folliclar B-cells express high levels of CD21 and CD1d and represent extrafollicular $IgM^{hi}IgD^{-}CD23^{-}CD21^{hi}CD1d^{hi}$ MZ B-cell precursors (Cariappa and Pillai, 2002). Due to selective stringency at many stages of development, only an estimated ~10% of

15-20 million immature B-cells produced each day in the bone marrow emerge as mature cells in the periphery (Cariappa and Pillai, 2002).

Terminally differentiated B-cells include plasmablasts, plasma cells, and memory cells and are found in lymphoid organ germinal centers or circulating. Plasmablasts express high levels of surface IgM, surface major histocompatability complex (MHC) II, and secrete high levels of IgM. Plasma cells express low levels of surface IgM, no surface MHC II, and secrete high levels of IgM. Development of plasma cells and plasmablasts is antigen (Ag) driven (Klein and Dalla-Favera, 2008). Especially important in regulating the production and appropriate folding of immunoglobulin proteins, endoplasmic reticulum (ER) resident folding factors (including X-box binding protein 1 (Xbp1)) and redox balance proteins are linearly upregulated until the end of plasma cell differentiation. IgM subunit expressions increase exponentially 2 days after initial Ag exposure, during the early induced innate immune response (van Anken et al., 2003).

### 1.1.1 Transcription Factors

Waves of lineage-specific transcription factors regulate B-cell terminal differentiation. The main regulatory transcription factors controlling B-cell terminal differentiation (Pax5, Blimp1, and Bcl6) compose a reciprocally inhibiting transcriptional 'switch.' Manipulation of mRNA levels or activation status of any one of these three transcription factors results in significant changes in gene expression as well as cell fate.

Initial entry of common lymphoid progenitors into the B-cell lineage depends on the appropriate expression of E2A, transcription factor COE1 (Ebf1), and Pax5 (Nutt and Kee, 2007; Cobaleda et al., 2007). Friend leukemia virus integration 1 (Fli1) positively regulates expression of E2A proteins (Zhang et al., 2008). E2A proteins, in concert with

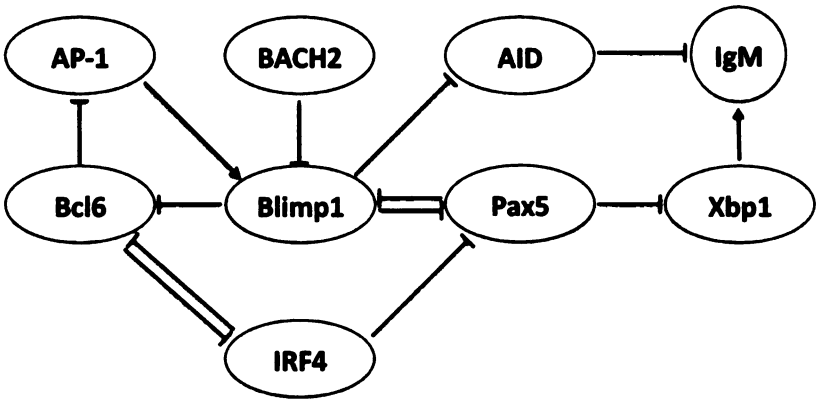



Figure 1.1.: **Schematic Representation of Reciprocal Repression of Transcription Factors Regulating IgM Expression in Differentiating B-cells.** Bcl6 inhibits AP-1 binding to the Blimp1 promoter and so represses its transcription. Blimp1 (a reciprocal repressor of Pax5 expression) is a transcription factor necessary for full induction of IgH and J chain and Xbp1 mRNA in B-cells. Xbp1 regulates the transcription of folding proteins as well as many B-cell receptor genes.

other transcription factors and signal transducer and activator of transcription 5 (STAT5) promote Ebf1 and c-Myc expression (Kee, 2009). Ebf1, in turn, promotes Pax5 and E2A expression. Pax5 promotes Ebf1 expression and represses Notch1 transcription, preventing T-cell fate in pro-B-cells (active Notch1 leads to E2A degradation and so inhibits B-cell transcription program maintenance) (Kee, 2009).

Ebf1 regulates Pax5 expression through chromatin remodeling of the entire Pax5 promoter region (Decker et al., 2009). The Pax5 promoter is also epigenetically regulated by DNA methylation in embryonic stem cells and mouse embryo fibroblasts. The Pax5 enhancer is demethylated upon the onset of hematopoeisis and organized into accessible chromatin at subsequent B-cell developmental stages (Decker et al., 2009).

Blimp1 (a reciprocal repressor of Pax5 expression) is a transcription factor necessary for full induction of IgH and J chain and Xbp1 mRNA in B-cells (Savitsky and Calame, 2006) (Figure 1.1). Upon activation, Blimp1 represses genes required for cell cycle entry,

DNA replication, and cell division (Savitsky and Calame, 2006). It is also a reciprocal repressor of Bcl6.

Bcl6 inhibits AP-1 binding to the Blimp1 promoter and so represses its transcription (Vasanwala et al., 2002) (Figure 1.1). Mice deficient in Bcl6 lack germinal centers in secondary lymphoid organs, indicating a complete repression of B-cell differentiation (Dent et al., 1997). Bcl6 co-repressor (Bcor) functions in concert with Bcl6 to repress transcription of many genes, potentially through epigenetic mechanisms such as histone methylation (Fan et al., 2009). These mechanisms direct gene silencing through chromatin modification by means of histone ubiquitination and demethylation (Gearhart et al., 2006).

When expressed, Pax5 works as a global regulatory element, controlling commitment to B-cell development by repressing B-lineage inappropriate genes and activating B-lineage specific genes. It is expressed exclusively from the pro-B-cell to mature B-cell stages and subsequently repressed during terminal plasma cell differentiation (Fuxa and Busslinger, 2007). Reprogramming induced by Pax5 transcriptional activity facilitates pre-B-cell receptor signaling, promotes B-lymphocyte differentiation, and regulates B-cell adhesion and migration (Delogu et al., 2006; Schebesta et al., 2007). Much of this reprogramming occurs as a result of Pax5 inhibiting the expression of Blimp1 (Mora-Lopez et al., 2007) (Shaffer et al., 2002) (Figure 1.1).

Xbp1 regulates the transcription of folding proteins as well as many B-cell receptor genes (Figure 1.1). Those B-cells lacking Xbp1 fail to secrete IgM (a marker indicative of successful plasma cell differentiation) (Masciarelli et al., 2010). Expression levels of Xbp1 reach high levels in primary splenic B-cells and B-cell lymphoma lines after 3 days of activation with LPS (van Anken et al., 2003; Calfon et al., 2002).

### 1.1.2 Cytokines

Most antigens are T-dependent, meaning that the B-cell requires T-cell help for maximal antibody production. Antigen cross linking to the BCR produces a signaling cascade modulated by spleen tyrosine kinase (Syk) and V-yes-1 Yamaguchi sarcoma viral related oncogene homolog (Lyn) (see section 1.1.7). Processed proteins from T-dependent antigens are presented on B-cell MHC II for recognition by $T_H2$ cells. The activated T-cell then secretes cytokines that activate the B-cell to trigger proliferation and differentiation into plasma cells. LPS activation induces $T_H1$ cytokines such as TNF-α, IFN-γ and IL-12 (Mukherjee et al., 2009) and production of interleukin-4 (IL-4) and interleukin-5 (IL-5) (Chiba et al., 2007). LPS-induced IL-4 production is TLR4 dependent, transcriptionally regulated, and requires *de novo* protein synthesis (Mukherjee et al., 2009). Splenocytes exposed to TCDD produce less IL-4 (responsible for stimulating activated B-cell and T-cell proliferation and upregulating MHC II production) and IL-5 (responsible for stimulating B-cell growth and increasing immunoglobulin secretion) (Nohara et al., 2002). The inhibition of IL-4 and 5 production induced by TCDD exposure may, in part, be responsible for the suppression of humoral immune response to LPS.

### 1.1.3 Calcium

A secondary signaling molecule with effects upon transcriptional elements, calcium is essential for effects elicited through BCR stimulation upon transcription factor expression including Pax5, Bcl6, MITF, Ets1, Fli1, interferon regulatory factor 4 (IRF4), Spi-B transcription factor (SpiB), and Blimp1 (Hauser et al., 2009). Calcium-loaded calmodulin inhibits DNA-binding of E2A, an inhibition that is essential for rapid down-regulation of immediate early genes after BCR activation (Saarikettu et al., 2004; Hauser et al.,

2009). Active (calcium-loaded) calmodulin also reduces Pax5, Bcl6, Fli1, and Ets1 mRNA expression and increases Blimp1 mRNA expression (Hauser et al., 2009).

The $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) Iα has been reported as a contributing factor to aryl-hydrocarbon receptor (AhR)-mediated genomic response (Monteiro et al., 2008b). However, more recent data indicates that the CaMKK inhibitor is an AhR ligand and that CaMK may not actually contribute to AhR signaling (Monteiro et al., 2008a). However, further reports conflict as to whether increases in internal calcium levels are induced through release of internal calcium stores (Puga et al., 1997) or external calcium influx via T-type channels (Kim et al., 2009) and whether this increase occurs immediately or after a period of time. Studies of TCDD catalytic degradation *in vitro* show that degradation is increased in the presence of calcium, functioning as a catalyst (Mitoma et al., 2006). For this reason, it is still likely that cells increase internal calcium levels in response to TCDD exposure in order to increase degradation of the toxicant, although whether the increase in cytosolic calcium is immediate or delayed may affect the efficiency of this process.

### 1.1.4 LPS Signaling

#### Stimulatory

The main receptor by which lipopolysaccharide (LPS) stimulates signaling in the cell is toll-like receptor 4 (TLR4). Soluble LPS binding protein (LBP) in the serum binds LPS and loads it onto cluster of differentiation 14 (CD14) in the plasma membrane. LPS-bound CD14 recruits the TLR4/lymphocyte antigen 96 (MD2) heterodimer and serves to initiate intracellular signaling through recruitment of cytoplasmic TIR domain containing adaptor proteins (TIRAPs) (Peng, 2005) (Figure 1.2). The TLR4/MD2 complex may

have additional discriminatory capability that confers specificity to the LPS recognition event, with B-cell lines and primary B-lymphocytes capable of differentiating between LPS chemotypes (Minguet et al., 2008). In fact, B-cell TLR4 may also recognize pathogenic antigens such as viral proteins or heat shock proteins (Peng, 2005). The role of toll-like receptors (TLRs) in B-cell activation and antibody response appears to be dependent on antigen type and stimulation context, with different LPS chemotypes and stimulation events leading to unique signaling responses (Lanzavecchia and Sallusto, 2007).

TLR4 stimulation leads to activation of two main cytoplasmic signaling cascades: TIRAP and TIR domain-containing adaptor-inducing interferon-β (TRIF)/TRIF-related adaptor molecule (TRAM). The activated cytoplasmic TIR domain of TLR4 recruits TIRAPs such as MyD88 and transduces signals through at least two signaling pathways. Through the DD, MyD88 recruits and activates IRAK which in turn recruits and activates TRAF6 in the plasma membrane. TRAF6 stimulates activation of TAK1, stimulating IKK (see section 1.1.5), JNK, and p38 activation (Figure 1.2).

The second signaling pathway triggered by LPS-mediated TLR4 stimulation initiates with formation of a TRIF/TRAM heterodimer. The TRIF/TRAM complex regulates interferon regulatory factor 3 (IRF3) activation and subsequent induction of type I interferons and co-stimulatory molecules (Peng, 2005). IRF3 in turn stimulates IFN-β transcription. Alternative association of TRIF with TRAF6 stimulates TRAF6 signaling as described above (Figure 1.2).

Unlike macrophages and dendritic cells, B-cells additionally utilize lymphocyte antigen 64, CD180 molecule (RP105)/lymphocyte antigen 86 (MD1) heterodimer (structurally related to TLR4/MD2) to recognize and respond to LPS (Peng, 2005). RP105/MD1 plays a uniquely important role in B-cells, enhancing TLR4-dependent LPS response (Peng, 2005; Kawai and Akira, 2006). Subsequent to LPS stimulation and through an unknown mecha-

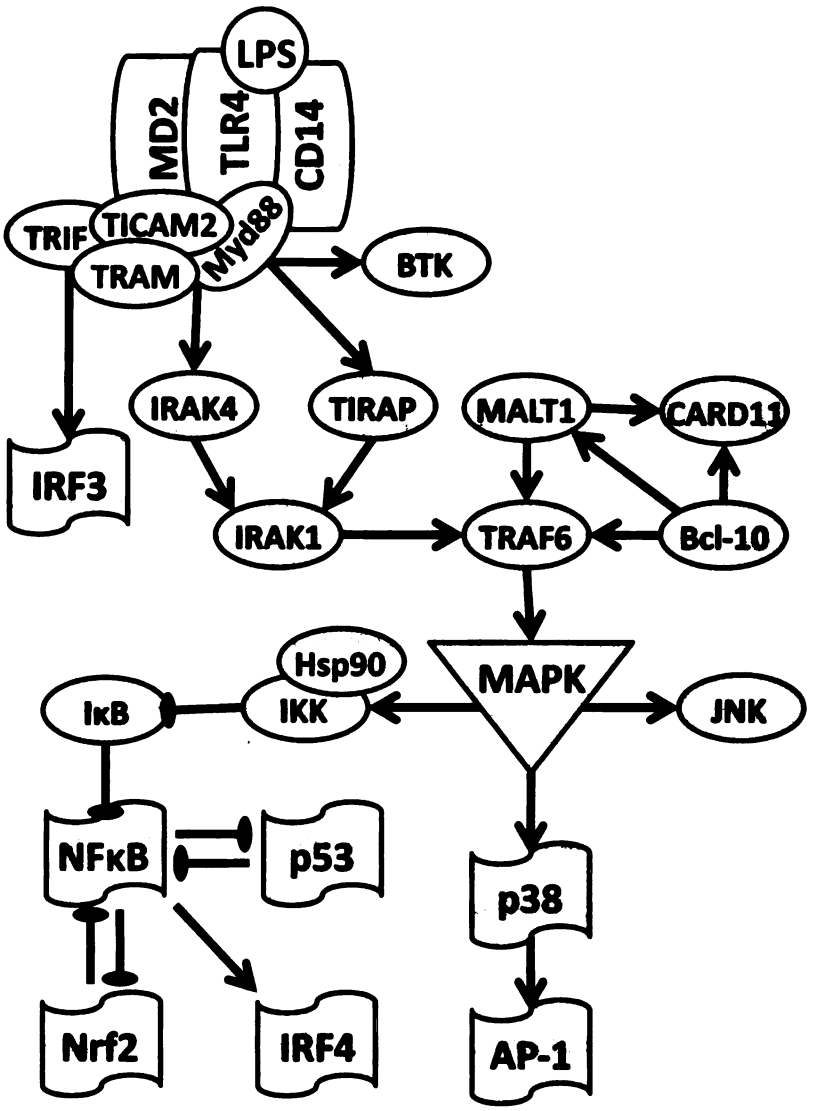



Figure 1.2.: **Signaling Occurring in B-cells as a Result of LPS binding to TLR4.** Soluble LBP binds LPS and loads it onto CD14 in the plasma membrane. LPS-bound CD14 recruits the TLR4/MD2 heterodimer and serves to initiate intracellular signaling through recruitment of cytoplasmic TIRAPs. The activated cytoplasmic TIR domain of TLR4 recruits TIRAPs such as MyD88 and transduces signals through at least two signaling pathways. Through the DD, MyD88 recruits and activates IRAK which in turn recruits and acivates TRAF6 in the plasma membrane. TRAF6 stimulates activation of TAK1, stimulating IKK (see section 1.1.5), JNK, and p38 activation.

nism, RP105 signaling activates phosphoinositide 3-kinase ($PI_3K$). $PI_3K$ catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) to form phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$), which recruits protein kinase B (Akt) and pyruvate dehydrogenase kinase (PDK) to the plasma membrane. Once recruited to the plasma membrane, Akt is phosphorylated and activated by PDK. Phosphatase and tensin homolog (PTEN) catalyzes the opposite reaction in which $PIP_3$ is dephosphorylated to form $PIP_2$. $PIP_2$ is degraded by phospholipase C (PLC) to form inositol triphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ is recognized by the inositol triphosphate receptor ($IP_3R$) to trigger release of internal calcium stores from the ER while DAG is capable of activating protein kinase C (PKC) to regulate Ras/Raf and MAPK signaling. PKC activation has also been shown to down-regulate DNA methylation activity in human lymphocytes, indicating a decrease in DNA methylation activity resulting from release of internal calcium stores (Bonilla-Henao et al., 2005) (Figure 1.3.

Through TLR4 alone, there exist two distinct signaling pathways stimulated by LPS binding with a third signaling pathway in B-cells that is also stimulated by LPS recognition. With so many pathways and interactions possible, it is small wonder that cells may have developed an increased ability to distinguish between LPS chemotypes and mount responses accordingly. The range of LPS chemotypes within a single preparation as well as the range of possible cellular responses must all be taken into consideration when studying a heterogeneous population such as isolated splenocytes.

**Attenuation**

Crucial to any cellular signaling cascade is appropriate regulation and attenuation. The TLR4-ligand response is attenuated by increased tumor protein 53 (p53) levels (Liu et al.,

2009a) and through a gradual decrease in plasma membrane TLR4 (Husebye et al., 2006). p53 is a transcription factor capable of transactivating genes with functions including cell cycle arrest, apoptosis, and metabolic changes (Green and Kroemer, 2009). In rat liver, TCDD attenuates p53 phosphorylation (and so transcriptional activity) by increasing extracellular signal-regulated kinase (ERK)-mediated murine double minute 2 oncogene (Mdm2) phosphorylation (a post-translational negative regulator of p53) (Paajarvi et al., 2005; Worner and Schrenk, 1996; Green and Kroemer, 2009).

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) directs the localization of LPS-bound TLR4 into endosomes-like structures after 1 hr of LPS stimulation (Husebye et al., 2006). This decrease in TLR4 at the plasma membrane is critical to regulate nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB) activation, with cellular Hrs knockdown eliciting a 70% increase in LPS-induced NFκB activation (Husebye et al., 2006). Without Hrs-mediated downregulation of TLR4 signaling, the cell may become hypersensitive to LPS-stimulus, producing large quantities of inflammation factors and inducing septic shock.

Increased levels of receptor-interacting serine-threonine kinase (RIP) down-regulate PTEN through NFκB-independent pathways (Park et al., 2009) and so increases the activation of Akt while decreasing the production of $IP_3$ and DAG from $PIP_3$. RIP is essential for the TAK1-dependent TLR4 activation of $PI_3K$ (Vivarelli et al., 2004) and suppresses forkhead box protein O (FoxO) transcription factors, so downregulaing cyclin-dependent kinase inhibitor 1B ($p27^{kip1}$) and favoring cell cycle progression (Park et al., 2009). However, the MyD88 mediated rapid NFκB activation and related inflammatory cytokine production is separate from the LPS-stimulated survival signals mediated by RIP in splenocytes (Vivarelli et al., 2004).

TLR4 gene expression is repressed by DNA methylation and histone deacetylation in the gene's 5' region (Takahashi et al., 2009), modifications that inhibit the recognition and binding of transcription factors through induction of a condensed chromatin conformation so repressing gene transcription. This may represent an epigenetic mechanism by which cells control sensitivity to an LPS stimulus.

### 1.1.5 NFκB Signaling

One of the signaling cascades which is crucial to cell signaling and survival, the NFκB pathway is stimulated through MAPK-mediated activation of IKK. Activated IKK induces IκB phosphorylation and degradation necessary for the release of NFκB. Functional IKK protein is crucial to B-cell survival, with conditional loss of IKK (α, β, or γ) resulting in decreased number of B-cells with impaired survival (Claudio et al., 2006). IκBα loss further results in an increased number of B-cells hyperresponsive to stimulation (Claudio et al., 2006). NFκB is a transcription factor, that when released from IκBα accumulates in the nucleus where it is able to affect transcription of many genes (Heiss et al., 2001) with constitutive activation inducing cell proliferation and apoptotic resistance (Amit and Ben-Neriah, 2003). Activation of NFκB negatively regulates PTEN transcription and p53 transcriptional activity yet is insufficient to promote survival of LPS-stimulated B-cells (Vivarelli et al., 2004).

### 1.1.6 Akt Signaling

Akt signaling is critical for survival and metabolic fitness (Patke et al., 2006) (Woodland et al., 2008). Appropriate Akt signaling prevents apoptosis through inhibition of Bim production, a process dependent upon mTOR (activated by Akt phosphorylation and inhibi-

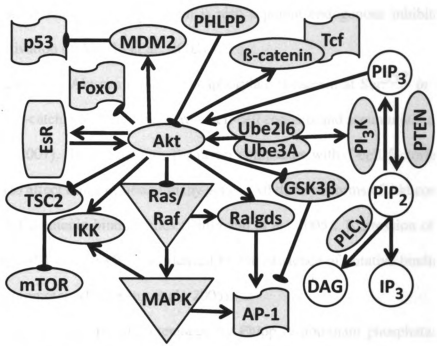



Figure 1.3.: **Schematic representation of Akt signaling.** Appropriate Akt signaling prevents apoptosis through inhibition of Bim production, a process dependent upon mTOR (activated by Akt phosphorylation and inhibition of TSC2, a negative regulator of mTOR). Akt is also capable of inactivating FoxO transcription factors, so inhibiting $p27^{kip1}$ transcription and regulating cell proliferation. Phlpp is a dominant phosphatase controlling cell cycle via specifically opposing Akt actions on $p27^{kip1}$ phosphorylation state

tion of TSC2, a negative regulator of mTOR) (Hahn-Windgassen et al., 2005) (Figure 1.3). Deregulation of Akt is highly associated with tumorigenesis in humans and mice (Blanco-Aparicio et al., 2010). Akt is also capable of inactivating FoxO transcription factors, so inhibiting $p27^{kip1}$ transcription and regulating cell proliferation (Medema et al., 2000) (Tran et al., 2003). Specifically, FoxO3 promotes p53 cytoplasmic accumulation and so cell survival (Green and Kroemer, 2009).

BCR crosslinking leads to Akt hyperphosphorylation and so activation (Ishiura et al., 2010). The BCR-induced activation of Akt requires $PI_3K$ and Syk (Li et al., 2002). IgM-bearing B-cells utilize B-cell scaffold protein with ankyrin repeats (Bank1) (a gene capable of limiting BCR-mediated Akt activation) to decrease signal strength and prevent a hyper-

IgM response (Aiba et al., 2006). Lyn is also a potent endogenous inhibitor of BCR-mediated activation of Akt (Li et al., 2002).

Upon activation, Akt can directly phosphorylate β-catenin at $Ser^{552}$ *in vitro* and *in vivo* causing β-catenin to disassociate from cell-cell contacts and accumulate in the nucleus (Fang et al., 2007). In the nucleus, β-catenin complexes with T-cell factor/transcription factor (Tcf) to affect transcription of many genes. All three isoforms of Akt contain at least 6 putative Tcf/β-catenin binding sites (Dihlmann et al., 2005). Expression of Akt is also regulated by AP-1 and NFκB, as evidenced by the existence of putative binding elements within their promoters (Dihlmann et al., 2005).

Akt signaling is specifically attenuated by Phlpp (a dominant phosphatase) isomers, with different Akt isomers individually targeted by Phlpp isomers. For example, Phlpp2 controls cell cycle via specifically opposing Akt3 actions on $p27^{kip1}$ phosphorylation state (Brognard and Newton, 2008).

### 1.1.7 BCR Signaling

The B-cell receptor (BCR) is composed of an immunoglobulin molecule in complex with, CD79a, and CD79b. Antigen binding to the immunoglobulin molecule induces signaling through the BCR to Syk and Lyn which activate Bruton's tyrosine kinase (Btk). Syk has been shown to be important in B-cell emigration (Cariappa and Pillai, 2002). Btk activity is increased upon BCR crosslinking and its deficiency results in reduced B-cell proliferation in response to LPS-stimulation (Baba et al., 2001). In the absence of Btk, most mature follicular B-cells fail to survive (Cariappa and Pillai, 2002). Activation of Src-family kinases such as Btk requires CD45 (Cariappa and Pillai, 2002). B-cells from

mice deficient in Lyn, Btk, $PI_3K$, BLNK, or phospholipase C $\gamma$ (PLC$\gamma$) exhibit impaired proliferative responses to LPS-stimulation (Yang and Desiderio, 1997).

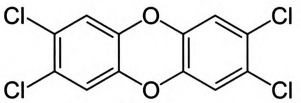
Similar to variegated responses occurring through TLR4 signaling, BCR signaling alters the fate of B-cell differentiation in response to different stimuli and environments. BCR inhibits some forms of B-cell differentiation, with increased BCR signaling decreasing differentiation of maturing B-cells into mid-zonal B-cells (Pillai and Cariappa, 2009) and persistant signaling abolishing LPS-induced plasma cell differentiation (Kurosaki, 1999). In other situations, strong BCR signaling favors follicular development (Pillai and Cariappa, 2009) and is required for maintenance of all peripheral B-cell populations (Cariappa and Pillai, 2002).

## 1.2 TCDD

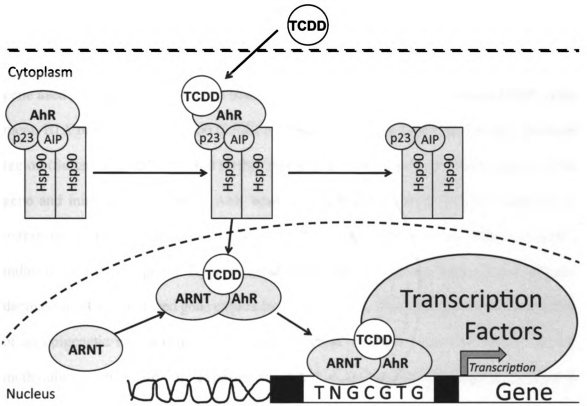
2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) is a widespread environmental contaminant with high lipophilicity, accumulating in the fat of carnivorous animals (including humans). TCDD is a polychlorinated dibenzodioxin and the most potent compound within its family. Mammalian effects of TCDD exposure include wasting, hepatotoxicity, cardiotoxicity, chloracne, death, and immunotoxicity. Specific immunologic aberrations observed in mice exposed to TCDD include the inability to mount an innate immune response to LPS challenge. For this reason, it is a useful compound in the evaluation of events necessary to the differentiation of B-cells.

### 1.2.1 TCDD Signaling

Cytoplasmic TCDD binds the AhR, which is commonly found in complex with AIP, p23, and 2 Hsp90 molecules. Binding of TCDD to the AhR induces release of all chaperone




(a) TCDD




(b) AhR Signaling

Figure 1.4.: **TCDD Signaling through the AhR.** Cytoplasmic TCDD binds the AhR, which is commonly found in complex with AIP, p23, and 2 Hsp90 molecules. Binding of TCDD to the AhR induces release of all chaperone proteins and translocation of the TCDD-bound AhR to the nucleus where it binds ARNT. In the nucleus, the AhR/ARNT dimer binds to AREs in the promoter regions of genes and regulates their transcription.

proteins and translocation of the TCDD-bound AhR to the nucleus where it binds ARNT and initiates transcription of several genes including Cyp1a1, NF-E2 related factor 2 (Nrf2), glutathione S-transferase, alpha 1 (Ya) (Gsta1), aldehyde dehydrogenase 3 family, member A1 (Aldh3a1), and epiregulin (EREG) (Nebert et al., 2000) (Figure 1.4). Phosphorylation of the charged linker region of Hsp90 modulates formation of a functional cytosolic AhR

complex (Puga et al., 2009). Although the chaperone protein complex maintains the AhR in a cytoplasmic state receptive to ligand binding, transcriptional functions of the AhR do not seem affected when the Hsp90 association with co-chaperone proteins is inhibited (Middendorp et al., 2003).

In the nucleus, the AhR/ARNT dimer binds to dioxin response elements (DREs) in the promoter regions of genes and regulates their transcription (Figure 1.4). The prototypical gene used to assess AhR-regulated transcription is Cyp1a1. Within 15min of TCDD treatment, E1A binding protein p300 (p300) is recruited to DREs within the Cyp1a1 promoter region (Sutter et al., 2009). p300 further serves to recruit other transcription factors to the gene and initiate transcription. AhR binding to DREs is reduced by DNA methylation within the gene enhancer region (Shen and Whitlock, 1989), so also reducing TCDD-induced gene transcription. Hypermethylation of the Cyp1a1 regulatory region causes a decrease in TCDD-induced gene expression (Okino et al., 2006), suggesting the possibility of an epigenetic mechanism in regulating response to TCDD exposure. Global hypermethylation upon embryonic exposure to TCDD is also accompanied by an increase in DNA methyl transferase activity (Wu and Sun, 2006), supplying additional evidence that TCDD may elicit epigenetic response within exposed cells.

Other transcription factors are also recruited to DREs along with the AhR. Estrogen receptor (EsR)$\alpha$ is recruited to AhR target genes in a TCDD-dependent manner and enhances target gene expression (MacPherson et al., 2009). Crosstalk occurring through the recruitment of EsR to DREs bound by activated AhR plays a role in tumor promotion (Matthews and Gstafsson, 2006). PKC inhibition blocks ligand-induced DNA-binding of AhR/ARNT heterodimer and leads to suppression of cytochrome P450, family 1 (Cyp1) gene expression (Puga et al., 2009).

After activation, AhR is quickly exported to the cytosol where it is degraded by the 26s proteasome, preventing constitutive receptor activity (Puga et al., 2009). The AhR is not required to generate a normal immune response, yet is obligatory in TCDD-induced immune response suppression (Vorderstrasse et al., 2001). Although immune response generation is normal in AhR-deficient animals, the doubling time of AhR-deficient cells is increased as compared to wild type (WT) cells (Ma et al., 2004). The functional AhR is not required for MAPK and PLC activation nor for JunB and c-Fos induction in response to TCDD (Puga et al., 2009) (Beebe et al., 1990), indicating that many important signaling pathways affected by TCDD exposure are regulated in a non-classical (AhR-independent) manner.

Some genes are expressed via a RelB/AhR transcriptional promoter recognition that does not require ARNT and involves signaling through the non-classical NFκB pathway (Matsumura, 2009). AhR activation also leads to time-dependent induction of BAFF, B-lymphocyte chemoattractant (BLC), chemokine (C-C motif) ligand 1 (CCL1) and IRF3 in human mϕs, inductions that require RelB (Vogel and Matsumura, 2009) and likely occur through crosstalk between the non-classical NFκB and the AhR pathways.

There is no evidence of direct DRE-mediated involvement in TCDD-induced alterations in Pax5 or Blimp1 regulation (Schneider et al., 2009). Instead, activated AhR downregulates AP-1 binding to the Blimp1 promoter region (Schneider et al., 2009). This may be the mechanism by which TCDD affects JunB and c-Fos induction even in cells without a functional AhR. Blimp1 binding to the Pax5 promoter is then suppressed by TCDD, resulting in decreased inhibition of Pax5 (Schneider et al., 2009).

Activated AhR also interacts with the RB/E2F axis (Puga et al., 2009) in order to suppress E2F transcriptional activity. Hyperphosphorylated retinoblastoma protein (RB) protein cannot adequately repress E2F activity (Puga et al., 2009), so increasing transcrip-

tion of cell cycle regulatory and DNA replication genes necessary for B-cell differentiation or tumor generation.

AhR activation further increases Nrf2 expression and subsequent binding to AREs (Yeager et al., 2009). In response to inflammation, a crosstalk may occur between Nrf2/ARE binding and NFκB signaling pathways (Prawan et al., 2008). Ablation of Nrf2 levels accelerates NFκB-mediated pro-inflammatory reactions (Li et al., 2008). The opposite is also true, in that Nrf2 activators attenuate LPS-induced NFκB activation (Li et al., 2008). Oxidants impede Nrf2 degradation, increasing its translocation to the nucleus, and likely reducing NFκB-mediated pro-inflammatory reactions through competition for binding to AREs (Li et al., 2008). LPS signaling through TLR4 exposure also induces oxidant stress through nitric-oxide generation, but the increase in oxidants due to LPS exposure induces septic shock and increases the incidence of septic shock (Zhang et al., 2000). Nrf2 signaling has thus been identified as a mechanism by which cells are protected from LPS-induced inflammatory response (Thimmulappa et al., 2006). However, when induced by TCDD exposure prior to LPS challenge the reduction in inflammatory response induced by Nrf2 signaling may be exaggerated to such an extent that instead of protecting against LPS-induced septic shock, it actually inhibits response to the challenge and so limits appropriate B-cell differentiation.

## 1.3 Epigenetics

Conrad Waddington originally defined epigenetics as the study of mechanisms by which genotypes give rise to phenotypes during development (Waddington, 1957). The definition was later modified by Arthur Riggs and colleagues to state "the study of mitotically and/or

meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996).

Epigenetic patterns of regulation can be passed from parental to filial generations of both cells and whole organisms. More transient occurrences are also identified in which various stages of cellular development and differentiation are regulated epigenetically, followed by complete erasure of the epigenetic mark(s) (Bird, 2007). Four specific forms of epigenetic regulation are small non-coding RNA, histone code, tissue-specific transcription factors (Liu et al., 2009b), and DNA methylation. Although these mechanisms are labeled and defined separately, no epigenetic mechanism functions in total isolation and the study of any in isolation will not fully evaluate the interactions occurring within the cell.

### 1.3.1 Small Non-coding RNA

Non-coding RNA's are functional RNA molecules that are not translated into protein. These RNA genes code for functionally important RNAs such as transfer RNA, ribosomal RNA, micro RNA (miRNA), and silencing RNA. Transfer and ribosomal RNA's are critical for transcription and translation processes in the cell. Through partial complement to messenger RNAs, miRNA downregulates the concentration of mature mRNA in higher order eukaryotic cells. Through an evolutionarily-conserved antiviral mechanism, double stranded RNA is recognized by the Dicer protein which digests the double stranded RNA into $\sim$20 nucleotide segments, thus inhibiting translation into protein (Csorba et al., 2009). siRNA's have recently been described as having longer lasting epigenetic effects than simple destruction of mRNA. These long lasting effects are mediated by DNA methylation of the targeted gene's promoter region resulting in gene silencing before mRNA transcription (Hawkins et al., 2009).

### 1.3.2 Histone Code

Histones associate with DNA and form nucleosomes composed of two copies each of core histones H2A, H2B, H3 and H4 and wrapped by 146bp of DNA with flexible N-terminus histone tail. Often, the tail is modified in the form of methylation, acetylation, ubiquitination, and/or phosphorylation in order to affect chromatin structure. Acetylated histones tend to preferentially associate with transcriptionally active chromatin (Hebbes et al., 1988). Several transcriptional activators such as Gcn5, p300/CBP, PCAF, TAF250, and the p160 family of nuclear receptor coactivators contain intrinsic histone acetyl-transferase activity which is required for their transcriptional regulatory activity (Roth et al., 2001; Wang et al., 1998). Histone methylation, unlike acetylation, remains relatively static and the precise regulatory role of histone methylation has not yet been elucidated (Zhang and Reinberg, 2001). Histone ubiquitination plays a critical role in regulating processes such as transcription, silencing, and DNA repair (Weake and Workman, 2008). Histone phosphorylation results in disparate regulation, with H2A phosphorylation resulting in mitotic chromosome condensation and H3 phosphorylation occurring after DNA-damaging events or correlating with gene activation (Grant, 2001). Formation of a condensed chromatin state causes genes to become inaccessible to transcription factors and DNA replication proteins.

### 1.3.3 Tissue-specific Transcription Factors

Often transcription factors are regulated in a developmental or tissue-specific manner. Recent evidence from the Tjian laboratory suggests that global changes in cell-type specific transcription may be facilitated by significant changes in general transcription factors such as TFIID, BAF, and Mediator (D'Alessio et al., 2009). The core transcription machinery,

that previously was assumed to operate in all cells, has been shown to be dramatically downregulated in some cell types while distinct transcription activating factors are thought to regulate distinct gene expression profiles (D'Alessio et al., 2009). Evidence suggests that tRNA and rRNA transcription, previously hypothesized to utilize TATA-binding protein (TBP) in the promoter recognition complex, may actually use related factors specific to the tissue in which the genes are transcribed. Disregulation of the appropriate expression of these cell-type specific transcription factors may result in inappropriate gene regulation and so differentiation or development.

### 1.3.4 DNA Methylation

DNA methylation, in the form of 5'-methylcytosine, normally occurs at ~70%-80% of CpG dinucleotides (Naveh-Many and Cedar, 1981a; Craig and Bickmore, 1994), a sequence particularly abundant in gene promoter regions (Kristensen and Hansen, 2009). CpG dinucleotide methylation protects healthy cells from inappropriate transcription of repetitive elements such as long interspersed nuclear elements (LINEs) and Alu repeats (Walsh et al., 1998) and may also help maintain chromosomal stability (Eden et al., 2003). Altered DNA-methylation, an epigenetic mechanism that plays a regulatory role in gene expression, has been proven to play multiple roles in carcinogenesis, development, and differentiation (Kurkjian et al., 2008) with unique methylomes observed in diverse cell types as well as gender-specific imprinting. A decrease in DNA-methylation results in reduced global histone deacetylase (HDAC) recruitment, decreased chromatin condensation, and increased gene expression (Martinowich et al., 2003). In addition to regulating the expression of genes, DNA methylation also reduces the mobility of transposons (Kato et al., 2003).

Four models of gene regulation through DNA methylation have been proposed:

1) **Direct** DNA-methylation: methylation in the promoter region of a gene inhibits binding of transcription factors while methylation in the intron(s) of a gene inhibits proper splicing (Mares et al., 2001; Decker et al., 2009)

2) DNA-methylation of an **enhancer's promoter**: methylation resulting in the increased binding of the enhancer to the genes promoter region and so increased transcription (Mares et al., 2001; Decker et al., 2009)

3) DNA-methylation of an **insulator region**: methylation upstream of an enhancer element results in neutralization of the insulator region and increased gene transcription (Hark et al., 2000)

4) Increase in DNA-methylation resulting in **global** recruitment of HDACs: induced condensed chromatin conformation reduces transcription factor access, so reducing gene expression (Baylin, 2005)

Methyl groups are transferred to and from DNA via DNA methyltransferase (DNMT)s and putative demethylases. All DNMTs use S-adenosyl methionine (SAM) as a methyl group donor in an $S_N2$ inversion reaction (Ulrey et al., 2005). DNMT1 is the must abundant mammalian DNA methyltransferase and is key to DNA methylation maintenance through copying methylation patterns on newly-duplicated hemi-methylated DNA. DNMT3 DNA methyltransferases methylate hemimethylated and unmethylated CpG regions. Specifically, DNMT3a and DNMT3b mediate *de novo* DNA methylation while DNMT3L is required for establishing maternal genomic imprints (Fatemi et al., 2002). In MZ and follicular B-cells, DNMT3a expression is differentially regulated.

DNA methyltransferases form a covalent bond between a cysteine residue in the enzyme's active site and a cytosine's C6 in DNA. Proper bond formation is followed by attack upon the methyl group of SAM. Proton abstraction from the DNA cytosine's C5

followed by β-elimination allows reformation of the C5-6 double bond and release of the enzyme and methylated DNA. While the methyl group is transferred to the DNA, the target cysteine is actually flipped out of line of the other nucleotides. When 5'-azacytidine is targeted by DNA methyltransferases, the saturated C5-6 bond mimics the transition state of cytidine formed prior to methyl transfer and the $sp^3$ character of C6 abrogates nucleophilic attack (Christman, 2002).

Treatment with 5'-azacytidine (an inhibitor of methyltransferase activity when incorporated into DNA or RNA during replication, causing global demethylation) results in almost complete abolishment of follicular B-cells while leaving MZ B-cells numbers relatively intact, indicating the importance of differential DNA methylation mechanisms and states in regulating the localization and development of B-cells within the spleen (Wang et al., 2006). Studies of the λ5-VpreB1 locus methylation status have discovered an increase in methylation status during the pre-B-cell stage, followed by an almost complete attenuation of all DNA methylation in mature B-cells (Szutorisz et al., 2005).

LPS challenge induces aberrant hypermethylation of hypermethylated in cancer-1 (Hic-1) exon 1a in mouse embryonic fibroblasts lacking p53 and (unchallenged) human follicular lymphomas (Tatemichi et al., 2008; Guo et al., 2005). This would indicate that hypermethylation of Hic-1 is important during rapid proliferation of cells. However, no further research suggesting altered DNA methylation in response to LPS challenge has been published. Because a clear genetic mechanism for crosstalk between LPS-regulated B cell differentiation and TCDD signaling has not yet been discerned, it is possible that this crosstalk may occur through epigenetic events.

Hypermethylation of the PTEN promoter in cerebral cavernous malformations has been linked to a significant downregulation in protein expression (Zhu et al., 2009). Increased

methylation of PTEN has also been associated with increased alterations in the $PI_3K$/Akt pathway and degree of tumor aggression in thyroid tumors (Hou et al., 2008).

## 1.4 Hypothesis

This work was designed to address two related but distinct hypotheses:

1) epigenetic events might, in part, underly B-cell differentiation to plasma cells

2) epigenetic events might, in part, underly TCDD-induced inhibition of LPS-induced B-cell differentiation

To test these two hypotheses, a number of investigations were undertaken to investigate the following sub-hypotheses:

1) DNA is differentially methylated in treated populations

2) DNA is differentially methylated within 10kb of genes

3) DNA methylation alters expression of selected genes

4) Genes exhibiting altered expression are important to B-cell differentiation

To assess the occurrence of potential epigenetic events, murine splenocytic DNA was probed via a non-biased AP-PCR, Capillary Electrophoresis (CE) method for RAMs where the DNA was hyper-, hypo-, or newly-methylated in comparison to DNA from control treated animals. This method is not limited by the regions already chosen on a PCR array and allows evaluation of total DNA for RAMs. PCR amplified DNA of the same length (in bp) as identified RAMs was sequenced and annotated to genes and CpG islands. Although this method precisely identifies potential RAMs, it is greatly limited by the ability of PCR products to be ligated into the plasmid and then amplified through clonal expansion of the host bacteria. Many promoters and enhancers with cis-acting regulatory abilities are located within 10kb of the transcriptional start site of genes, leading to the hypothesis that should

a RAM occur within this 10kb it may affect the regulatory capabilities of the enhancer and promoter regions. A distance of 10kb up- and downstream of the sequenced PCR products was examined for gene coding sequences, miRNA coding sequences, and repeat elements. In this way, alterations in DNA methylation may have effects on gene expression were directly observed.

Alterations in the pattern and sites of DNA methylation were identified in all treatment groups. Of particular interest were those RAMs identified within 10kb of (a) transcription factor-, (b) LINE repeat elements and (c) histone modifier-encoding sequences. When coding sequences are differentially methylated, it is reasonable to hypothesize that the expression patterns may also be altered. Therefore, through analysis of alterations in DNA methylation, this work describes new evidence indicating that many different epigenetic regulatory mechanisms may affect the differentiation of B-cells and the toxicity of TCDD.

RAMs were identified as a result of LPS challenge that were different (in size, location, and methylation state) from those identified as a result of TCDD exposure. Interestingly, over the course of preliminary evaluation, it became evident that the RAMs identified in response to LPS-mediated activation or TCDD exposure were not identical to those identified in response to LPS+TCDD treatment. This evidence suggests mechanisms regulating maintenance of the DNA-methylome is altered in LPS-induced signaling, TCDD exposure, and through a novel crosstalk mechanism occurring in concomitant treatment.

# 2. DNA METHYLATION: A POTENTIAL MECHANISM OF CROSSTALK OCCURRING IN MURINE SPLENOCYTES EXPOSED *IN VIVO* TO LIPOPOLYSACCHARIDE (LPS) AND 2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)

McClure, E.A., North, C.M., Kaminski, N., and Goodman, J.I.

a manuscript in preparation for submission to *Toxicological Sciences*, 2010

## 2.1 Abstract

B cells within the splenocyte population, isolated from mice treated with lipopolysaccharide (LPS), differentiate into antibody-producing plasma cells *in vivo*. Pretreatment with 2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) inhibits differentiation. We hypothesize that altered DNA methylation, an epigenetic event, plays a key role in this inhibition. DNA was isolated from splenocytes prepared 6 days post experiment initiation from 5-6wk old female C57BL/6 mice dosed with: TCDD, 3 or 30μg/kg, on day 0; or LPS, 25μg/mouse, on day 4; or sequentially with TCDD and then LPS on days 0 and 4, respectively. To discern regions of altered DNA methylation (RAMs), DNA was restricted with HpaII (a methylation sensitive enzyme), followed by arbitrarily primed PCR and capillary electrophoresis. LPS, 3, or 30μg/kg TCDD alone resulted in 40, 43 and 42 RAMs, respectively, while LPS challenge subsequent to 3 or 30μg/kg TCDD resulted in 34 and 39 RAMs, respectively. Interestingly, the combined treatment lead to 4 RAMs seen with LPS alone, 4 RAMs seen with TCDD alone, and 19 RAMs observed only in the co-treatment group. These data indicate treatment with LPS or TCDD can alter DNA methylation in splenocytes. The combined TCDD+LPS leads to RAMs that are unique: we do not simply observe the sum of the RAMs that form from the individual treatments. PCR products representing a number

of the RAMs observed were cloned, sequenced and annotated. Those unique to the LPS-TCDD combination include: Bank1 (involved in B cell receptor-induced Ca2+ mobilization); Adcy5 (membrane-bound calcium-inhibitable adenylyl cyclase); Arvcf (involved in protein-protein interactions); CtBP2 (corepressor targeting diverse transcription regulators), Lingo2 (leucine-rich repeat and immunoglobulin-like domain-containing nogo receptor-interacting protein 2; Pctk3 (may play a role in signal transduction cascades); Krr1 (involved in nucleolar processing of pre-18S ribosomal RNA and ribosomal assembly). Unique RAMs in the TCDD+LPS group indicate crosstalk between the actions of LPS to induce B-cell differentiation and aryl-hydrocarbon receptor (AhR) signaling which, presumably, mediates the inhibitory effect of TCDD. To our knowledge, this is the first largescale evaluation of changes in DNA methylation due to LPS exposure and the first identification of a DNA methylation-based crosstalk occurring in splenocytes exposed to TCDD prior to LPS-challenge.

## 2.2 Introduction

TCDD is a widespread environmental contaminant with high lipophilicity and severe immunosuppressive effects. Specific immunologic aberrations observed in mice exposed to TCDD include the inability to mount an innate immune response to LPS challenge (Kerkvliet, 2002).

LPS is a bacterial endotoxin and potent B-cell mitogen capable of stimulating the innate humoral immune response in animals. The innate immune response occurs within 4 hrs of initial infection, with induced innate response occurring 4-96 hrs post initial infection. Upon stimulation with an antigen, B cells in peripheral lymphoid organs activate and begin rapidly proliferating, thereby increasing expression of surface and secreted immuno-

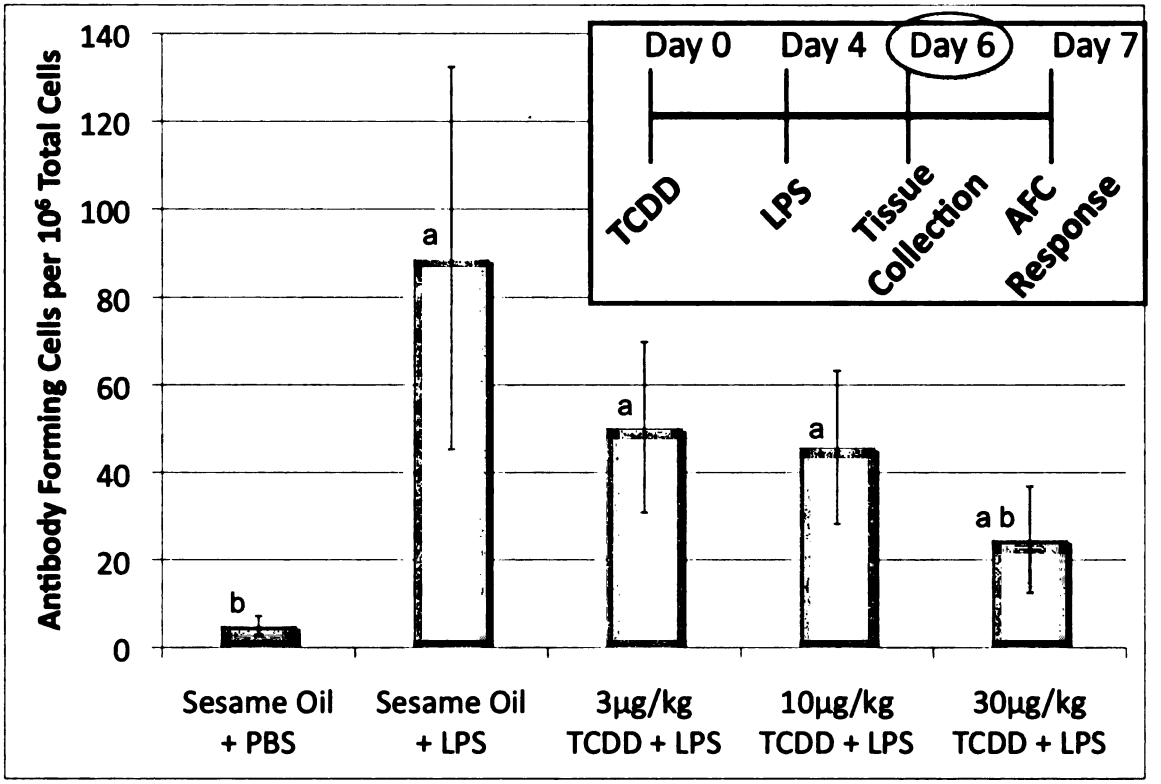



Figure 2.1.: **AFCs as a Function of Treatment with LPS and TCDD.** All samples are from the same animals as used in (North et al., 2009). 6 female C57BL/6 mice per treatment group were treated on day 0 via oral gavage with 0, 3, 10, or 30$\mu$g/kg TCDD; mice were further treated on day 4 via intraperitoneal injection with 0 or 25$\mu$g LPS in PBS; combined treatment included mice treated on days 0 and 4 as described above. AFCs was measured on day 7. Data are depicted ± standard error. a. significantly different from control. b. significantly different from LPS treatment. **Insert:** Timeline showing treatment administration, splenic tissue collection, and AFCs response measurement. Day 6 is highlighted as the day on which this study's samples were obtained. Figure modified from data reported in (North et al., 2009)

globulin M (IgM) (Dooley and Holsapple, 1988). A lack of increased IgM expression and secretion is indicative of B-cell activation and suppression of proliferation in response to LPS exposure.

Murine TCDD-induced immunological suppression is observed both *in vivo* and *in vitro* (North et al., 2009) and is easily measured as a suppression of IgM secretion. Maximum *in vitro* suppression of primary sheep erythrocyte IgM response observed at 30$\mu$g/kg TCDD in C57BL/6 mice (Figure 2.1) (Vecchi et al., 1980). Immune suppression in splenocytes

treated *in vivo* with TCDD+LPS is also accompanied by decreased B-lymphocyte-induced maturation protein 1 (Blimp1) expression, decreased $CD19^{+}$ cells, decreased expression of immunoglobulin J (IgJ), κ, and $\mu$ chains, and decreased major histocompatability complex (MHC) class II expression (North et al., 2009).

Genomic effects of TCDD in animals are mediated through binding to the AhR. Upon TCDD binding, AhR releases all chaperone proteins [aryl-hydrocarbon receptor interacting protein (AIP), heat shock protein co-chaperone p23/prostaglandin E synthase 3 (p23), and 2 heat shock protein 90kDa (Hsp90)] and translocates from the cytoplasm to the nucleus. Here it binds aryl-hydrocarbon nuclear translocator (ARNT) and functions as a transcriptional regulator binding aryl-hydrocarbon response element (ARE). The ARNT/AhR complex upregulates transcription of NF-E2 related factor 2 (Nrf2) (Yeager et al., 2009), which inhibits nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB) activation.

Upon systemic challenge, LPS binding to toll-like receptor 4 (TLR4) stimulates an intracellular signaling cascade terminating in the phosphorylation of interferon regulatory factor 4 (IRF4) (Takeda and Akira, 2004). IRF4 indirectly increases Blimp1 expression by inhibiting B-cell lymphoma 6 protein (Bcl6) and paired box protein 5 (Pax5) expression. Together Bcl6, Blimp1, and Pax5 create a reciprocally repressing transcription factor 'switch' which terminates in Pax5's inhibiting IgM production. The 'switch hypothesis' anticipates an increase in IRF4 activity (as expected post TLR4 stimulation) to decrease Bcl6 and Pax5 transcription, thus upregulating Blimp1 transcription and IgM expression.

TCDD-induced inhibition of the primary humoral immune response likely occurs upstream of antibody (IgM) production (North et al., 2009). Specifically, recently published evidence indicates negative regulation of IgM production resulting from failure of TCDD-exposed splenocytes to upregulate Blimp1 expression (North et al., 2009). In which stage of upstream signaling pathways TCDD inhibits Blimp1 expression is not well understood.

In 1989, Shen et al. published data suggesting DNA-methylation within the cytochrome P450, family 1, subfamily A, polypeptide 1 (Cyp1a1) promoter reduces the response to TCDD exposure (Shen and Whitlock, 1989). Epigenetic mechanisms of regulation, such as DNA methylation, occur through the addition of mitotically and/or meiotically heritable changes to gene function that do not entail changes in DNA sequence (Wu and Sun, 2006). Epigenetic regulation can be transient, as identified in cellular development and differentiation. DNA methylation, in the form of 5'-methylcytosine, normally occurs at 40-80% of CpG dinucleotides, with non-CpG methylation occurring as up to 55% of total cell methylation (Naveh-Many and Cedar, 1981a; Dupont et al., 2009; Ramsahoye et al., 2000). An increase in DNA-methylation results in the recruitment of histone deacetylase (HDAC) and induction of condensed chromatin conformation that decreases transcription factor binding, thus reducing gene expression. A decrease in DNA-methylation results in reduced HDAC recruitment, decreased chromatin condensation, and increased gene expression (MacDonald and Roskams, 2009).

LPS challenge has been proven to induce aberrant hypermethylation of hypermethylated in cancer-1 (Hic-1) exon 1a in mouse embryonic fibroblasts lacking tumor protein 53 (p53) (Tatemichi et al., 2008). However, to the best of our knowledge, no further research suggesting altered DNA methylation in response to LPS challenge has been published.

We hypothesize that signaling due to LPS, TCDD, or combined treatments is regulated, in part, by altered DNA methylation. To test this hypothesis, we have assessed the methylation status of splenocyte DNA treated *in vivo* with LPS, TCDD and LPS+TCDD. The genomically unbiased method chosen includes arbitrarily primed PCR followed by capillary electrophoresis, sequencing of PCR products, annotation to genes, and pathway analysis. This method has allowed the identification of many region of altered DNA methylation (RAM)s resulting from LPS, TCDD, and TCDD+LPS treatments. Specifically, we have

demonstrated the occurrence of DNA methylation crosstalk, wherein the methylation status of combined treatments is different from that in either treatment alone, when mice are concomitantly treated with TCDD and LPS. Annotated genes include many important for cell survival, apoptosis, and B-cell signaling. Cumulatively, these observations suggest a significant role of DNA methylation in regulating LPS and TCDD effects *in vivo* and suggest a role for DNA methylation in the TCDD-induced inhibition of B-cell stimulation by LPS.

## 2.3 Materials and Methods

### 2.3.1 Preparation of *in vivo* Splenocyte Samples

#### Chemicals

TCDD was purchased from Accustandard (New Haven, CT) and prepared in sesame oil (Sigma-Aldrich, St. Loius, MO). *Salmonella typhosa* LPS (Sigma-Aldrich, St. Louis, MO) was prepared in PBS immediately prior to administration.

#### Animals

Mice, treatments, and splenocyte collection were described previously (North et al., 2009). The same splenocyte samples from those animals sacrificed upon day 6 (post LPS exposure) in the previous study (North et al., 2009) were used for this study. Female 6-8 week old C57BL/6 mice were purchased from the National Cancer Institute and housed in accordance with Michigan State University Institutional Animal Care & Use Committee policy. On day 0, TCDD (0, 3, 10, or 30$\mu$g/kg in sesame oil) was administered by single oral gavage. On Day 4, to initiate primary humoral immune response, LPS (0 or 25$\mu$g

in PBS) was administered by intraperitoneal injection. Spleen samples were collected on days 4-7 from all treatment groups (6 animals per group). Splenocytes were mechanically disrupted to form single-cell suspension and stored at -80°C. Mice from day 4 that received only TCDD or vehicle treatment, were used to establish baseline TCDD effects (measured by AFCs response from samples collected on day 7) (Figure 2.1).

### 2.3.2 Evaluation of DNA Methylation Status by AP-PCR and CE

Changes in DNA methylation status were evaluated using an Arbitrarily Primed PCR (AP-PCR) and Capillary Electrophoresis (CE) procedure (Bachman et al., 2006). This technique permits evaluation of genomic RAMs including hypomethylations (less methylation than that observed in control), hypermethylations (more methylation than that observed in control), and new methylations (methylation not observed in control) simultaneously. Most importantly, the procedure is unbiased in that it does not involve an evaluation of preselected genes but rather all genomic regions targeted by the restriction enzymes and arbitrary primer.

#### DNA Isolation

Single cell splenocyte suspensions were removed from -80°C and mixed with 1mL, 4°C TRIzol® Reagent (Sigma-Aldrich, St. Louis, MO) before homogenizing completely using a Dounce homogenizer. DNA was isolated according to the manufacturers (Sigma-Aldrich, St. Louis, MO) protocol and precipitated with ethanol before dissolution in NaOH/HEPES buffer (pH=8.4) and storage at -20°C.

## Restriction Digest

Each isolated DNA sample was subjected to double restriction digestion performed in duplicate as previously described (Bachman et al., 2006). Preliminary digestion with a methylation-insensitive enzyme, RsaI, ensures complete digestion by the methylation-sensitive enzyme, HpaII. RsaI recognizes 5'-GTAC-3' sites and cuts between the guanine and adenine, but will not restrict DNA if the external cytosines (5' and 3') are methylated. HpaII recognizes 5'-CCGG-3' sites and cuts between the internal cytosine and guanine when unmethylated.

## AP-PCR and CE Analysis of DNA Products

AP-PCR and CE were performed as described previously (Phillips and Goodman, 2009).

## Data Analysis

PCR products were evaluated with regard to size (in base pairs) and corresponding peak areas as measured by CE. An average peak area was calculated for each PCR product in treatment groups and compared to that of the control group. Regions of altered methylation (RAMs) were identified as DNA regions in treatment groups with PCR products significantly (as determined by Students two-tailed t-test, $p \leq 0.05$) different in area than that of the control group. RAMs include: a) complete hypomethylations (i.e. 100% decrease from methylation status observed in control) and partial hypomethylations (significant decrease in methylation when compared to control); b) hypermethylations (significant increase in methylation when compared to control); and c) new methylations (PCR product

formed in treatment that did not form in control). A detailed description of the data analysis procedure was provided previously (Bachman et al., 2006).

**Carry Forward and Unique RAMs**

One-way analysis of variance (ANOVA) was performed to compare RAMs (occurring at the same PCR product size in $\geq$2 treatment groups). Common RAMs with the same change in methylation status (one-way ANOVA, $p \leq 0.05$) in treatment groups were identified as Carry Forward RAMs. Unique RAMs include: 1) RAMs exhibiting different extents of methylation change in the same direction (one-way ANOVA, $p \leq 0.05$); 2) RAMs in common, which exhibited opposite directional changes; and 3) RAMs observed in only 1 treatment group.

### 2.3.3 Cloning and Annotation of RAMs

**Cloning and Sequencing of AP-PCR Products**

AP-PCR products were electrophoresed through a 3% High Resoluion agarose gel (Sigma-Aldrich, St. Loius, MO). PCR products were excised and DNA was isolated using Ultrafree-DA Columns (Millipore, Billerica, MA), and used for cloning reactions prepared with the pGEM-T Easy Vector System (Promega, Madison, WI) and *Escherichia coli* JM109 competent cells (Promega, Madison, WI). Clones that contained PCR product inserts were purified and sequenced using T7 sequencing primers as outlined in pGEM-T Easy Vector Technical Manual (Promega, Madison, WI) at the Research Technology and Support Facility (Michigan State University, East Lansing, MI) using an ABI 3730xl Genetic Analyzer.

**Comparison of the sizes of cloned and sequenced AP-PCR products to the sizes of RAMs**

For sequenced inserts, the sizes of cloned products were compared with the sizes of identified RAMs as described previously (Phillips and Goodman, 2009). Six animals per experimental group were used and restriction digestions performed in duplicate, followed by AP-PCR, for a total of 12 reactions.

**RAM Annotation to Genes**

BLAST like alignment tool (BLAT) database searches (UCSC Genome Browser, July 2007 mouse assembly, http://genome.ucsc.edu/cgi-bin/hgBlat?command=start &org=mouse) determined in which regions of the genome sequenced RAMs occurred. RAMs were classified according to a scheme (Figure S.1) that indicates location in relation to a gene (e.g., within an intron, overlapping an exon, overlapping the transcriptional start site, or within 10kb of a gene). Genes identified as being within 10kb of a RAM are referred to as annotated genes. RAMs were also categorized by chromosomal location and gene function (Table S.2).

**PCR Products Annotating to $\geq$2 RAMs**

Those PCR products of size (as measured post-sequencing) within 2bp of both a carry forward and unique RAM could not be definitively labeled and are designated uncertain (CF/U). For example: the 237bp PCR product sequenced from 30$\mu$g/kg TCDD+LPS treatment could annotate to a 239bp carry forward new-methylation or a 238bp unique new-methylation and is designated as uncertain.

### DAVID and GO Analysis of Annotated Genes

The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (Huang et al., 2008) was used to investigate the functions of annotated genes. With this program, Gene Ontology (GO) Ashburner et al. (2000) information is efficiently examined for all genes annotating to particular cell processes. Major processes examined were apoptosis, calcium ion storage, cell cycle, differentiation, proliferation, chromatin modification, innate immunity, ion homeostasis and transport, kinase activity, protein transport, oxidoreductase activity, transcription, ubiquitin cycle, and vesicle-mediated transport (Tables 2.3 and S.3).

### Pathway Analysis of Annotated Genes

Pathway Studio 6.0® (Ariadne Genomics, Rockville, MD) was used to investigate the functions of annotated genes. With this program, common targets (genes, functional classes, and cellular processes affected by $\geq$2 genes annotated to RAMs) are identified. Treatments with 30$\mu$g/kg TCDD were used because this dose exhibited significant inhibition of the primary IgM antibody response (Figure 2.1). Pathway Studio 6.0® was also utilized to uncover documented links between RAMs and B-cell or epigenetic processes including: chromatin remodeling, gene silencing, receptor internalization, mitogenesis, mRNA splicing and stabilization, NO biosynthesis, WNT signaling, differentiation, programmed cell death, protein degradation and folding, receptor mediated endocytosis, transcription initiation, xenobiotic clearance, cell development and fate, cell migration, DNA damage recognition, calcium ion homeostasis, DNA recombination, oxidative stress, translation, DNA replication, apoptosis, and survival (Figures 2.3 and 2.4).

## 2.4 Results

### 2.4.1 RAM Identification

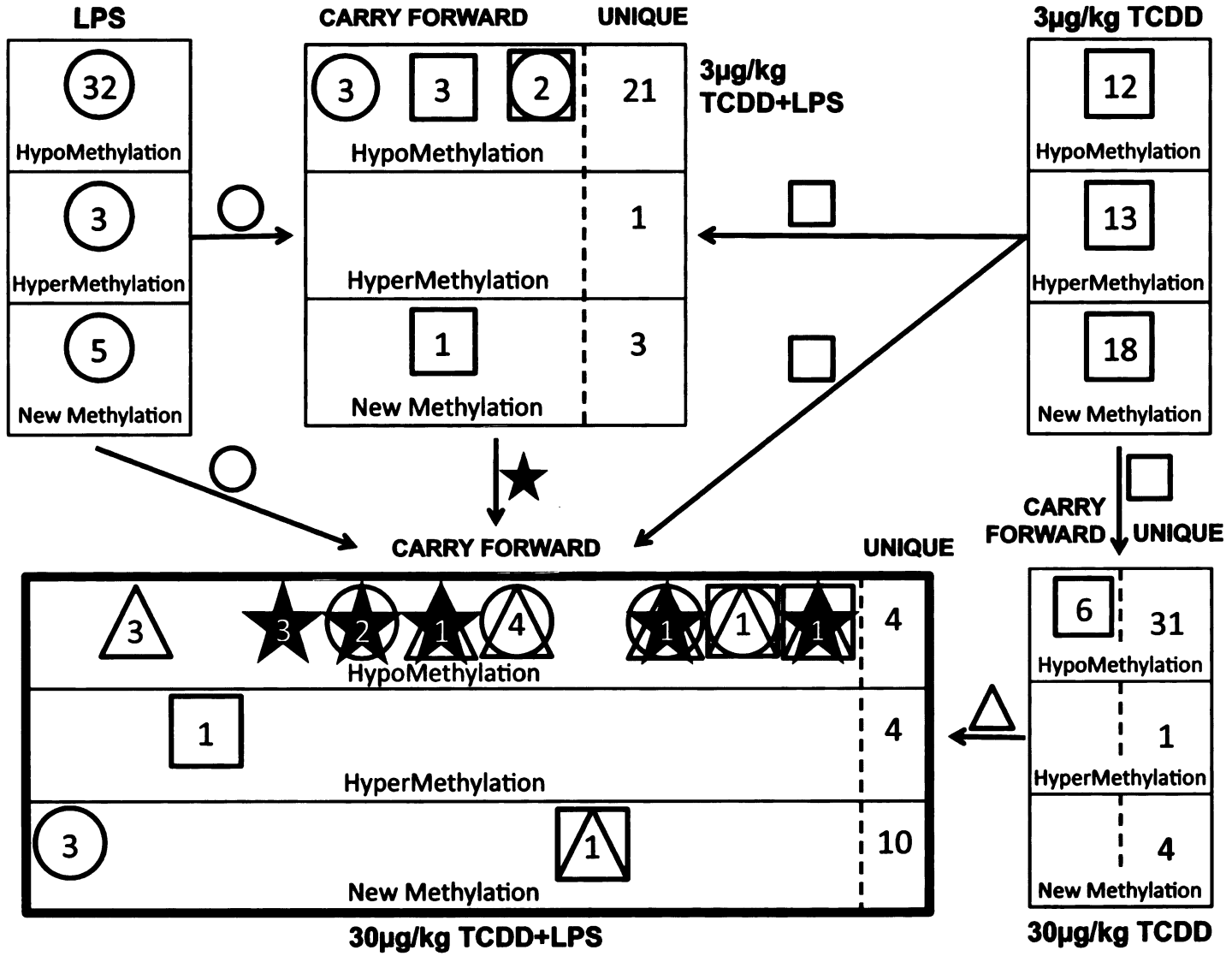
Hypo-, hyper-, and newly methylated RAMs were observed in splenocytes from all female C57BL/6 mice treated with (0, 3, or 30μg/kg) TCDD and (0 or 25μg) LPS when compared to those mice treated with only vehicle. Many individual RAMs occurred in more than one treatment group as carry forward RAMs, i.e., observed at both doses of LPS or TCDD, or observed following treatment with LPS or TCDD and also seen in an LPS+TCDD treatment (Figure 2.2).

Treatment with LPS resulted in 32 hypo-, 3 hyper-, and 5 new-methylations. Treatment with 3μg/kg TCDD resulted in 12 hypo-, 13 hyper-, and 18 new- methylations while treatment with 30μg/kg TCDD resulted in 37 hypo-, 1 hyper-, and 4 new-methylations. RAMs that carried forward from 3 to 30μg/kg TCDD were limited to 6 hypomethylations. The 1 hyper- and all 4 newly methylated RAMs observed in 30μg/kg TCDD were unique to that treatment (Figure 2.2).

Treatment with 3μg/kg TCDD+LPS resulted in 29 hypo-, 1 hyper-, and 4 new-methylations. RAMs that carried forward from a single treatment group to 3μg/kg TCDD+LPS include 3 hypo- (LPS), 3 hypo- (3μg/kg TCDD), and 1 new-methylation (3μg/kg TCDD+LPS). RAMs that carried forward from two treatment groups to 3μg/kg TCDD+LPS were 2 hypomethylations (LPS and 3μg/kg TCDD). A number of RAMs unique to 3μg/kg TCDD+LPS were identified: 1 hyper-, 21 hypo-, and 3 new-methylations. Of these unique RAMs, many were identified as having a different methylation status in LPS or 3μg/kg TCDD treatments: 3 unique hypomethylations in 3μg/kg TCDD+LPS were new-methylations in 3μg/kg TCDD, 6 unique hypo- and 1 unique new-methylation in 3μg/kg TCDD+LPS were hypermethylations in 3μg/kg TCDD, 1 unique new-methylation

in 3μg/kg TCDD+LPS was a hypomethylation in both LPS and 3μg/kg TCDD, 1 unique new methylation in 3μg/kg TCDD+LPS was a hypomethylation in LPS, and the 1 unique hypermethylation in 3μg/kg TCDD+LPS was a hypomethylation in both LPS and 3μg/kg TCDD (Table S.2 and data not shown). Of the 12 remaining unique hypomethylated RAMs observed in 3μg/kg TCDD+LPS, none were the same size as RAMs observed in LPS or 3μg/kg TCDD (Figure 2.2).

Figure 2.2. : **RAMs in Splenocytes.** RAMs were discerned in DNA of splenocytes isolated from mice treated with: LPS (25$\mu$g/mouse), TCDD (3 or 30$\mu$g/kg), or LPS+TCDD as indicated. Carry Forward (occurring in two or more treatment groups) and Unique (occurring in only one treatment group) RAMs were observed in 30$\mu$g/kg TCDD, 3$\mu$g/kg TCDD+LPS, and 30$\mu$g/kg TCDD+LPS treatments. RAMs carried forward from 3$\mu$g/kg TCDD (□), LPS (◯), 3$\mu$g/kg TCDD+LPS (★), and 30$\mu$g/kg TCDD (△) to 30$\mu$g/kg TCDD, 3$\mu$g/kg TCDD+LPS, and 30$\mu$g/kg TCDD+LPS. RAMs representing hypo-, hyper-, and new-methylations are depicted separately


LPS
CARRY FORWARD
UNIQUE
3µg/kg TCDD
32
HypoMethylation
3
HyperMethylation
5
New Methylation
3
3
2
21
HypoMethylation
1
HyperMethylation
1
3
New Methylation
3µg/kg TCDD+LPS
12
HypoMethylation
13
HyperMethylation
18
New Methylation
CARRY FORWARD
UNIQUE
CARRY FORWARD
UNIQUE
3
3
2
1
4
1
1
1
4
HypoMethylation
1
4
HyperMethylation
3
1
10
New Methylation
30µg/kg TCDD+LPS
6
31
HypoMethylation
1
HyperMethylation
4
New Methylation
30µg/kg TCDD

Treatment with 30μg/kg TCDD+LPS treatment resulted in 20 hypo-, 5 hyper-, and 14 new-methylations. RAMs that carried forward from a single treatment group to 30μg/kg TCDD+LPS include 3 new- (LPS), 1 hyper- (3μg/kg TCDD), 3 hypo-(30μg/kg TCDD), and 3 hypomethylations (3μg/kg TCDD+LPS). RAMs that carried forward from two treatment groups to 30μg/kg TCDD+LPS include 2 hypo- (LPS and 3μg/kg TCDD+LPS), 4 hypo- (LPS and 30μg/kg TCDD), 1 hypo- (30μg/kg TCDD and 3 μg/kg TCDD+LPS), and 1 new-methylation (3μg/kg TCDD and 30μg/kg TCDD). RAMs that carried forward from three treatment groups to 30μg/kg TCDD+LPS include 1 hypo- (LPS, 3μg/kg TCDD, and 30μg/kg TCDD), 1 hypo- (LPS, 30μg/kg TCDD, and 3μg/kg TCDD+LPS), and 1 hypomethylation (3μg/kg TCDD, 30μg/kg TCDD, and 3μg/kg TCDD+LPS). A number of RAMs unique to 30μg/kg TCDD+LPS were identified including 4 hypo-, 4 hyper-, and 10 new-methylations. Of these unique RAMs, many hypermethylations were identified as having a methylation status different from that observed in LPS, 3μg/kg TCDD, 30μg/kg TCDD, or 3μg/kg TCDD+LPS treatments: 2 were hypomethylations in both LPS and 30μg/kg TCDD, and 1 was a hypomethylation in LPS (Table S2 and data not shown). Of the remaining unique RAMs observed in 30μg/kg TCDD+LPS, 4/4 hypo-, 1/4 hyper-, and 10/10 new-methylations were not the same size as RAMs observed in LPS, 3μg/kg TCDD, 30μg/kg TCDD, or 3μg/kg TCDD+LPS (Figure 2.2).

### 2.4.2 RAM Annotation

RAMs were annotated to sequenced PCR products within 2bp of RAM size. Treatment with LPS resulted in 40 RAMs, of which 63% were annotated to genes: 100% (3/3) of hyper-, 59% (19/32) of hypo-, and 80% (4/5) of new-methylations. Treatment with 3μg/kg TCDD resulted in 43 RAMs, of which 56% were annotated to genes: 54% (7/13)

of hyper-, 58% (7/12) of hypo-, and 56% (10/18) of new-methylations. Treatment with 30$\mu$g/kg TCDD resulted in 42 RAMs, of which 64% were annotated: 100% (1/1) of hyper-, 59% (22/37) of hypo-, and 100% (4/4) of new-methylations. Treatment with 3$\mu$g/kg TCDD+LPS resulted in 34 RAMs, of which 62% were annotated to genes: 69% (20/29) of hypo- and 25% (1/4) of new-methylations. Treatment with 30$\mu$g/kg TCDD+LPS resulted in 39 RAMs, of which 78% were annotated: 100% (5/5) of hyper-, 73% (17/23) of hypo-, and 79% (11/14) of new-methylations. Although a large percentage of RAMs were annotated, the chosen procedure limited complete annotation due to incomplete transfection. The probability also exists that those sequences found in one treatment group but not another may actually derive from both while not successfully transforming in both. However, to limit the number of sequences examined, we have chosen to limit annotation only to those sequences of the same size sequenced in the same treatment group(s) in which the RAM was originally observed (Figure 2.2 and Tables 2.1-2.2, S.2).

Table 2.1 : **Solo treatment annotated genes**

[a]Of the 40 RAMs identified in LPS treatment, 63% were annotated: 100% (3/3) of hyper-, 59% (19/32) of hypo-, and 80% (4/5) of new-methylations

[b]Of the 43 RAMs identified in 3$\mu$g/kg TCDD treatment, 56% were annotated: 54% (7/13) of hyper-, 58% (7/12) of hypo-, and 56% (10/18) of new-methylations

[c]Of the 42 RAMs identified in 30$\mu$g/kg TCDD treatment, 64% were annotated: 100% (1/1) of hyper-, 59% (22/37) of hypo-, and 100% (4/4) of new-methylations

[d]RAM methylation status is indicated as: a) Hypomethylation (significant decrease in methylation when compared to control), b) hypermethylation (significant increase in methylation when compared to control), or c) new-methylation (PCR product formed only in treatment)

| | LPS[a] | | 3μg/kg TCDD[b] | | 30μg/kg TCDD[c] | |
|---|---|---|---|---|---|---|
| | Gene | NCBI RefSeq | Gene | NCBI RefSeq | Gene | NCBI RefSeq |
| Hypomethylations[d] | Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Acyp2 | NM_029344 |
| | Cldn18 | NM_019815 | Clic6 | NM_172469 | Ap2a1 | NM_001077264 |
| | Clic6 | NM_172469 | E2F8 | NM_001013368 | Atg7 | NM_028835 |
| | Clstn2 | NM_022319 | Pgm2 | NM_028132 | Cldn18 | NM_019815 |
| | Ddx54 | NM_028041 | Th | NM_009377 | Clic6 | NM_172469 |
| | E2F8 | NM_001013368 | | | Clstn2 | NM_022319 |
| | Krr1 | NM_178610 | | | Col4a5 | NM_007736 |
| | Lima1 | NM_001113545 | | | Ddx54 | NM_028041 |
| | Myof | NM_001099634 | | | E2F8 | NM_001013368 |
| | Ntrk2 | NM_001025074 | | | Fbxl7 | NM_176959 |
| | Pgm2 | NM_028132 | | | FOlr4 | NM_176807 |
| | Prickle2 | NM_001081146 | | | Il1rapl1 | NM_001160403 |
| | Six3 | NM_011381 | | | Il3ra | NM_008369 |
| | Tcf4 | NM_009333 | | | Krr1 | NM_178610 |
| | Th | NM_009377 | | | Large | NM_010687 |
| | Ubac2 | NM_026861 | | | Lima1 | NM_001113545 |
| | | | | | Lingo2 | NM_175516 |
| | | | | | Luzp2 | NM_178705 |
| | | | | | Myof | NM_001099634 |
| | | | | | Nostrin | NM_181547 |
| | | | | | Npt1 | NM_009198 |
| | | | | | Npt4 | NM_134069 |
| | | | | | Pgm2 | NM_028132 |
| | | | | | Prickle2 | NM_001081146 |
| | | | | | Six3 | NM_011381 |
| | | | | | Spbc25 | NM_025565 |
| | | | | | Tcf4 | NM_009333 |
| | | | | | Th | NM_009377 |
| | | | | | Ubac2 | NM_026861 |
| | | | | | Unc5c | NM_009472 |
| | | | | | Usp13 | NM_001013024 |
| | | | | | Wbp1 | NM_016757 |
| Hyper-methylations[d] | Krr1 | NM_178610 | Anapc7 | NM_019805 | | |
| | Lrrc56 | NM_153777 | Bank1 | NM_001033350 | Grm2 | NM_001160353 |
| | Qsox1 | NM_001024945 | Crabp1 | NM_013496 | Zfp128 | NM_153802 |
| | Rassf7 | NM_025886 | Ralgds | NM_009058 | Zscan22 | NM_001001447 |
| | Six3 | NM_011381 | Theg | NM_011583 | | |
| | Tha1 | NM_027919 | | | | |
| New-methylations[d] | | | Bcl7c | NM_009746 | | |
| | | | Ddx54 | NM_028041 | | |
| | | | Glul | NM_008131 | | |
| | Srms | NM_011481 | Me9 | NM_178243 | Cdh4 | NM_009867 |
| | Zfp128 | NM_153802 | Prickle2 | NM_001081146 | Sgpp2 | NM_001004173 |
| | Zscan22 | NM_001001447 | Taf5l | NP_598727 | Taf4a | NM_001081092 |
| | | | Teddm1 | NM_178244 | | |
| | | | Xlr5a | NM_001045539 | | |
| | | | Xlr5c | NM_031493 | | |

Table 2.2 : **Combined treatment annotated genes**[a]

[a]Cloning and subsequent sequencing of arbitrarily primed PCR products annotated 79% (82/104) of identified RAMs. Detailed data (including PCR product size and those that do not annotate to genes) available in Table S.2

[b]RAMs observed in 3$\mu$g/kg TCDD+LPS and 30$\mu$g/kg TCDD+LPS treatment are divided into Carry Forward (CF-observed in TCDD and/or LPS treatment alone as well as TCDD+LPS treatment), Unique (U-observed only in TCDD+LPS treatment), and Carry Forward or Unique (observed as either CF or U). Annotated genes are identified as Carry Forward or Unique when the sequenced PCR product is within 2bp of both a Carry Forward and a Unique RAM

[f]Of the 34 RAMs identified in 3$\mu$g/kg TCDD+LPS treatment, 62% were annotated: 69% (20/29) of hypo- and 25% (1/4) of new-methylations

[g]Of the 39 RAMs identified in 30$\mu$g/kg TCDD+LPS treatment, 78% were annotated: 100% (5/5) of hyper-, 73% (17/23) of hypo-, and 79% (11/14) of new-methylations

| | Hypomethylations[b] | | | | Hypermethylations[b] | | New-Methylations[b] | |
|---|---|---|---|---|---|---|---|---|
| | 3μg/kg TCDD+LPS[c] | | 30μg/kg TCDD +LPS[d] | | 30μg/kg TCDD +LPS[d] | | 30μg/kg TCDD+LPS[d] | |
| | Gene | NCBI RefSeq | Gene | NCBI RefSeq | Gene | NCBI RefSeq | Gene | NCBI RefSeq |
| Carry Forward[e] | | | Acyp2 | NM_029344 | | | | |
| | | | Atg7 | NM_028835 | | | | |
| | | | Clic6 | NM_172469 | | | | |
| | | | Col4a5 | NM_007736 | | | | |
| | | | Ddx54 | NM_028041 | | | | |
| | | | Fbxl7 | NM_176959 | | | | |
| | | | FOlr4 | NM_176807 | | | | |
| | | | Il1rapl1 | NM_001160403 | | | | |
| | | | Il3ra | NM_008369 | | | Glul | NM_008131 |
| | | | Large | NM_010687 | | | Me9 | NM_178243 |
| | | | Luzp2 | NM_178705 | | | Srms | NM_011481 |
| | | | Nostrin | NM_181547 | | | Teddm1 | NM_178244 |
| | | | Npt1 | NM_009198 | | | | |
| | | | Npt4 | NM_134069 | | | | |
| | | | Spbc25 | NM_025565 | | | | |
| | | | Ubac2 | NM_026861 | | | | |
| | | | Unc5c | NM_009472 | | | | |
| | | | Usp13 | NM_001013024 | | | | |
| | | | Wbp1 | NM_016757 | | | | |
| Unique[e] | Acyp2 | NM_029344 | | | | | | |
| | Arl10 | NM_019968 | | | | | | |
| | Atg7 | NM_028835 | | | | | | |
| | Cltb | NM_028870 | | | | | | |
| | Col4a5 | NM_007736 | | | | | | |
| | Fbxl7 | NM_176959 | | | | | | |
| | Higd2a | NM_025933 | | | Arvcf | NM_033474 | | |
| | Il1rapl1 | NM_001160403 | | | Btbd11 | NM_028709 | | |
| | Krr1 | NM_178610 | | | Cnga2 | NM_007724 | | |
| | Large | NM_010687 | | | Ehbp1l1 | NM_053252 | | |
| | Lingo2 | NM_175516 | Adamts17 | NM_001033877 | Gjd4 | NM_153086 | Acvr1b | NM_007395 |
| | Lipc | NM_008280 | Arl10 | NM_019968 | Jarid1a | NM_145997 | Arl16 | NM_197995 |
| | Luzp2 | NM_178705 | Clstn2 | NM_022319 | Kcnk7 | NM_010609 | Cadps2 | NM_153163 |
| | Nop16 | NM_178605 | Cltb | NM_028870 | Map3k11 | NM_022012 | Ccdc137 | NM_152807 |
| | Nostrin | NM_181547 | Higd2a | NM_025933 | Pcnxl3 | NM_144868 | Hgs | NM_008244 |
| | Npt1 | NM_009198 | Lingo2 | NM_175516 | Prickle2 | NM_001081146 | Il17rd | NM_134437 |
| | Npt4 | NM_134069 | Myof | NM_001099634 | Qsox1 | NM_001024945 | Pde6g | NM_012065 |
| | Pkd1l2 | NM_029686 | Nop16 | NM_178605 | Six3 | NM_011381 | Taf5l | NP_598727 |
| | Poldip3 | NM_178627 | | | Slc11a2 | NM_008732 | | |
| | Rrp7a | NM_029101 | | | Slc38a9 | NM_178746 | | |
| | Serhl | NM_023475 | | | Ube2l6 | NM_019949 | | |
| | Six3 | NM_011381 | | | | | | |
| | Spbc25 | NM_025565 | | | | | | |
| | Tcf4 | NM_009333 | | | | | | |
| | Ubac2 | NM_026861 | | | | | | |
| | Unc5c | NM_009472 | | | | | | |
| | Usp13 | NM_001013024 | | | | | | |
| CF or U[e] | Ap2a1 | NM_001077264 | | | | | | |
| | Clic6 | NM_172469 | | | | | | |
| | Ddx54 | NM_028041 | Ntrk2 | NM_001025074 | | | Ap1b1 | NM_007454 |
| | E2F8 | NM_001013368 | Prickle2 | NM_001081146 | | | Zfp128 | NM_153802 |
| | Pgm2 | NM_028132 | | | | | Zscan22 | NM_001001447 |
| | Prickle2 | NM_001081146 | | | | | | |

One PCR product (187bp) annotated to a repeat element (L1Md_F2) located on multiple chromosomes. BLAT searches also revealed 33 PCR products that annotated to regions further than 10kb from known gene(s). The methylation status of 31 of these 33 RAMs was unambiguous and could be classified as hypo- (19 RAMs), hyper- (6 RAMs), or new- (6 RAMs) methylations. The remaining 2 RAMs exhibited a different methylation status depending upon treatment: the 356bp RAM was hypermethylated in 3$\mu$g/kg TCDD and newly methylated in 3$\mu$g/kg TCDD+LPS and 30$\mu$g/kg TCDD; the 370bp RAM was hypomethylated in LPS and 30$\mu$g/kg TCDD and newly methylated in 3$\mu$g/kg TCDD (Table S.2).

### 2.4.3 DAVID and GO Analysis

DAVID analysis identified few biological processes enriched by annotated genes. For this reason, an assessment of similar GO terms with which annotated genes associated was performed. Biological processes associated with annotated genes highlighted specific differences between treatment groups. For example: LPS annotated genes do not associate with apoptosis, innate immunity, or ubiquitin cycle; 30$\mu$g/kg TCDD annotated genes associate with apoptosis, innate immunity, and ubiquitin cycle; 30$\mu$g/kg TCDD+LPS annotated genes do not associate with proliferation but do associate with apoptosis, chromatin modification, innate immunity, and ubiquitin cycle (Tables 2.3 and S.3).

### 2.4.4 Annotated Gene Interaction Analysis

Pathway Studio 6.0® interaction analysis revealed common (potentially affected by two or more genes of interest) target genes and gene functional classes for annotated genes in LPS, 30$\mu$g/kg TCDD, and 30$\mu$g/kg TCDD+LPS treatments (Figure 2.3). Specific treat-

Table 2.3: **Functional Groups Represented by Annotated Genes**[a]

| Signaling Pathway or Cellular Process | Treatment Groups[b] | | | | |
|---|---|---|---|---|---|
| | LPS | 3μg/kg TCDD | 3μg/kg TCDD+LPS | 30μg/kg TCDD | 30μg/kg TCDD+LPS |
| Apoptosis[c] | – | ✔ | ✔ | ✔ | ✔ |
| Calcium Ion Storage | ✔ | – | ✔ | ✔ | ✔ |
| Cell Cycle[d] | ✔ | ✔ | ✔ | ✔ | ✔ |
| Cell Differentiation | ✔ | ✔ | ✔ | – | ✔ |
| Cell Proliferation | ✔ | ✔ | ✔ | ✔ | – |
| Chromatin Modification | – | – | – | – | ✔ |
| Innate Immunity[e] | – | ✔ | ✔ | ✔ | ✔ |
| Ion[f] | ✔ | ✔ | ✔ | ✔ | ✔ |
| Kinase[g] | ✔ | ✔ | ✔ | – | ✔ |
| Protein Transport[h] | ✔ | ✔ | ✔ | ✔ | ✔ |
| RedOx[i] | ✔ | ✔ | ✔ | ✔ | ✔ |
| Transcription[j] | ✔ | ✔ | ✔ | ✔ | ✔ |
| Ubiquitin[k] | – | – | ✔ | ✔ | ✔ |
| Vesicles[l] | ✔ | ✔ | ✔ | ✔ | ✔ |

[a] Signaling pathway and cellular process involvement discerned via GO analysis, genes are grouped based on (single or many related) GO terms. Full list of each treatment groups annotated genes presented in Table S.3

[b] Treatment groups are marked as having at least one representative annotated gene (✓) or none (-)

[c] Genes involved in (positive and negative) regulation of apoptosis

[d] Genes involved in the cell cycle and mitosis

[e] Genes involved in B-cell activation and innate immune response

[f] Genes involved in calcium ion homeostasis, ion transport, and metal ion binding

[g] Genes involved in protein kinase binding and kinase activity

[h] Genes with kinase and phosphatase activity

[i] Genes involved in protein transport, intracellular protein transport, and protein transporter activity

[j] Genes using or producing secondary signaling molecules including $Ca2^{+}$, cAMP, and cGMP

[k] Genes involved in oxidation reduction and oxidoreductase activity

[l] Genes involved in transcription, transcription repressor activity, and transcription from RNA polymerase II promoter

[m] Genes involved in ubiquitin thiolesterase activity, regulation of protein ubiquitination, and ubiquitin-protein ligase activity

[n] Genes involved in endocytosis, exocytosis, and vesicle-mediated transport

ment interactions were further examined to identify those interactions that occurred in TCDD or LPS treatments but not TCDD+LPS treatments or those interactions occurred in TCDD+LPS but not TCDD or LPS treatments (Figure 2.4). A number of potential regulatory interactions were identified as occurring in LPS but not in 30μg/kg TCDD+LPS (Figure 2.4B) as well as many occurring in 30μg/kg TCDD+LPS that did not occur in LPS (Figure 2.4A).

In LPS treatment, many annotated genes are involved in important signaling pathways such as WNT, MAPK, and PI3K-AKT (Figure 2.3). Most of these same regulated genes and processes are also evident in 30μg/kg TCDD+LPS treatment. However, LPS RAMs annotated to two genes (Tcf4 and Grm2) to which 30μg/kg TCDD+LPS RAMs did not annotate. Tcf4 may be responsible for regulating WNT, VEGF, and Akt while Grm2 may be responsible for regulating MAPK (Figure 2.4B).

In 30μg/kg TCDD+LPS treatment, many annotated genes are involved in important signaling genes and pathways such as caspase, GAP, JNK-Jun/Fos, MAPK, PI3K-AKT, Ras, SMAD, and WNT. Annotated genes carried forward from LPS to 30μg/kg TCDD+LPS include Ap1b1, Ddx54, Glul, Ntrk2, Pdzd2, and Six3, which potentially regulate caspase, GAP, Jun, MAPK, PI3K-Akt, Ras, and WNT signaling. Annotated genes carried forward from 30μg/kg TCDD to 30μg/kg TCDD+LPS include Atg7, Ddx54, Glul, Il3ra, Pdzd2, and Six3, which potentially regulate Jun, PI3K-Akt, and WNT signaling. Annotated genes unique to 30μg/kg TCDD+LPS include Acvr1b, Hgs, Il17rd, Map3k11, and Pde6g, which potentially regulate caspase, GAP, JNK-Jun/Fos, MAPK, PI3K-AKT, Ras, and SMAD signaling. In fact, the only signaling pathway regulated by carry forward but not unique 30μg/kg TCDD+LPS annotated genes is WNT (Figure 2.4).

A number of annotated genes are regulated in different directions in multiple treatment groups. For example: phosphoinositide 3-kinase ($PI_3K$) is upregulated by a LPS carry

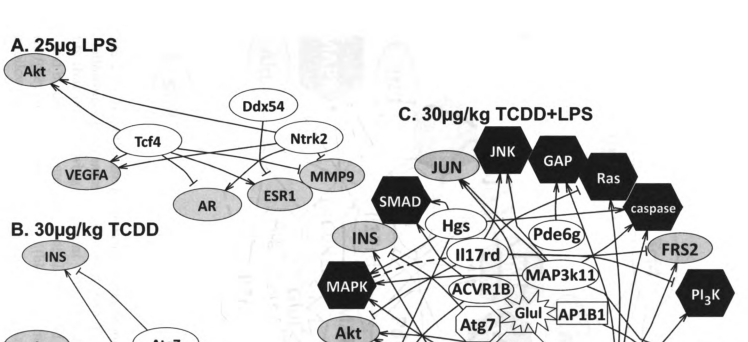



Figure 2.3.: **Common Targets of Annotated Genes.** Putative positive (→), negative (⊣), and binding (- - -)interactions of all annotated genes (white) with common genes (grey) and/or pathways (black) are diagrammed. Genes and pathways are considered common targets if they are potentially regulated by one or more annotated genes (listed in Table S2). Annotated genes from (C) 30$\mu$g/kg TCDD+LPS treatment are identified as Unique (◯) or Carry Forward from (A) 25$\mu$g LPS (□), (B) 30$\mu$g/kg TCDD (⬭), or both 25$\mu$g LPS and 30$\mu$g/kg TCDD (☆). References for interactions are presented in Figures S.2-S.4 and Table S.5

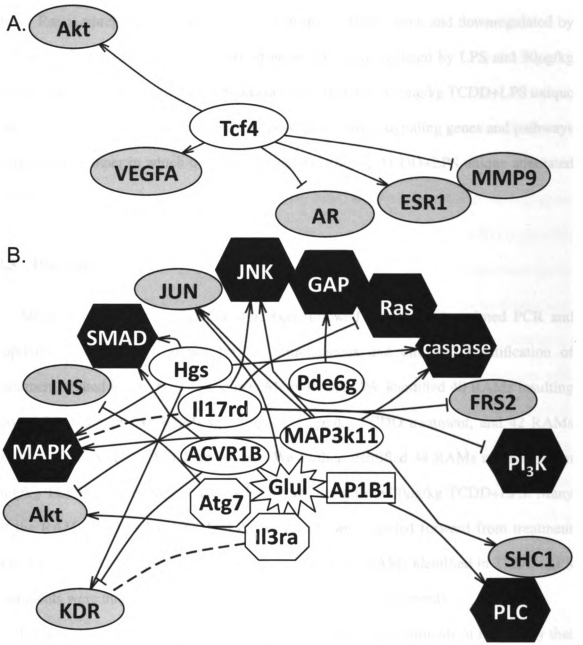



Figure 2.4.: **Hypothesized regulatory interactions in splenocytes following LPS (but not following LPS treatment combined with TCDD) and 30$\mu$g/kg TCDD+LPS treatments (but not following treatment with LPS alone).** Putative positive (→), negative (⊣), and binding (- - -)interactions of all annotated genes (white) with common elucidated genes (grey) and functional classes (black) are diagrammed. A. Interactions only occurring following 30$\mu$g/kg TCDD+LPS treatment. Annotated genes are identified as Unique or Carry Forward from LPS (□), TCDD (○), or both LPS and TCDD (☆). B. Interactions only occurring following 25$\mu$g LPS treatment. References for interactions supplied in Figures S.2-S.4 and Table S.5.

forward annotated gene and downregulated by a 30$\mu$g/kg TCDD+LPS unique annotated gene; Ras is upregulated by a LPS carry forward annotated gene and downregulated by a 30$\mu$g/kg TCDD+LPS unique annotated gene; Akt is upregulated by LPS and 30$\mu$g/kg TCDD carry forward annotated genes and downregulated by a 30$\mu$g/kg TCDD+LPS unique annotated gene. Other carry forward annotated genes affect signaling genes and pathways in the same manner in which they are affected by 30$\mu$g/kg TCDD+LPS unique annotated genes.

## 2.5 Discussion

Methylation-sensitive restriction digestion followed by arbitrarily primed PCR and capillary electrophoresis allows for the simultaneous and unbiased identification of treatment-related hypo-, hyper-, and new Methylations. We identified 40 RAMs resulting from LPS treatment, 43 RAMs resulting from 3$\mu$g/kg TCDD treatment, and 42 RAMs resulting from 30$\mu$g/kg TCDD treatment. We further identified 34 RAMs resulting from 3$\mu$g/kg TCDD+LPS treatment and 39 RAMs resulting from 30$\mu$g/kg TCDD+LPS. Many of the RAMs identified in TCDD+LPS treatments were carried forward from treatment with LPS and TCDD alone. However, almost half of the RAMs identified in TCDD+LPS treatments were unique (not carried forward from any other treatment).

Because so many RAMs were unique to the TCDD+LPS treatments, it is unlikely that the phenomenon is random. We therefore propose that the methylation patterns observed in mice treated with LPS are a means by which the splenocytes regulate gene expression. We further propose that TCDD exposure may alter the normal splenocyte methylation pattern and interferes with processes by which normal LPS-induced methylation patterns arise. The interference of TCDD upon processes directing LPS-induced methylation patterns

results in a unique methylation pattern observed subsequent to either treatment alone. These results indicate the possibility of crosstalk occurring between TCDD and LPS treatments to uniquely affect methylation status of various DNA sequences. Some RAMs were also observed as having a different methylation status between treatment groups, indicating interference and differing influences upon methylation processes between treated groups (Figure 2.2).

AP-PCR products, identified as RAMs, were subsequently sequenced to identify genes to which these RAMs annotated. Annotated genes include many involved in B cell signaling, differentiation, and proliferation. DAVID and GO analysis identified annotated genes involved in proliferation were affected by treatment with LPS but not by treatment with TCDD+LPS or TCDD. DAVID and GO analysis of annotated genes also identified genes involved in apoptosis and cytoskeletal rearrangement that are affected by treatment with TCDD+LPS but not by treatment with LPS. These results are consistent with that observed *in vivo* by North et al. where LPS-injection increased the total number of B-cells producing IgM in sheep red blood cell (SRBC) assay, a result that is decreased by concomitant exposure to TCDD. Results from this study are consistent with those reported by North et al and indicate that changes in methylation may regulate some of the proliferative changes observed in mice treated *in vivo* with LPS and TCDD.

Further analysis of annotated genes for potential interactions via Pathway Studio 6.0® elucidated common targets between treatment groups. LPS annotated genes included two genes (Tcf4 and Ddx54) that were not annotated from 30μg/kg TCDD+LPS treatment. Tcf4 positively affects Vegfa, inhibits AR, and inhibits Mmp9. These are interactions that occur only in response to LPS treatment and not to any treatment containing 30μg/kg TCDD. Ddx54 negatively affects ESR1 which is an interaction that occurs in LPS and 30μg/kg TCDD treatments but not in 30μg/kg TCDD+LPS treatment. Annotated genes

from LPS treatment inhibit Mmp9 and positively affect Vegfa. Regulation of these genes is observed only in LPS treatment and not in any other examined treatment. Jun and Shc1 are positively affected by treatment with 30$\mu$g/kg TCDD+LPS but are not affected in any other examined treatment. Treatment with 30$\mu$g/kg TCDD+LPS resulted in annotated genes that potentially regulate common targets MAPK, SMAD, JNK, GAP, Ras, caspase, PI3K, and PLC signaling pathways. These pathways are critical in intracellular balance between cellular differentiation/proliferation and apoptosis, a balance regulated by LPS stimulation and deregulated upon concomitant exposure to TCDD. Those annotated genes and signaling pathways that Pathway Studio 6.0® has identified are prime candidates for cross-talk between LPS and TCDD signaling. Due to the large number of potential locations of interaction, the signaling occurring in LPS, TCDD, and TCDD+LPS treatments is complicated and likely is affected by many more extra/intracellular factors (Figures 2.3, 2.4, and 3.2).

Protein kinase C (PKC) activation has previously been shown to down-regulate DNA methylation activity in human lymphocytes, indicating a decrease in DNA methylation activity resulting from release of internal calcium stores (Bonilla-Henao et al., 2005). Decreased methylation was observed in all three treatment groups, potentially indicating that PKC activity is increased in these splenocytes whether through calcium release (as occurs in LPS signaling) or other mechanisms.

Results of this study provide compelling evidence for a DNA methylation based cross-talk between LPS and TCDD signaling in splenocytes. Because many annotated genes are closely involved in B-cell differentiation, we propose that alterations in DNA methylation in splenocytes also occur in B-cells and induce the genes expression changes implicated in controlling plasma cell differentiation and previously described by North et al. Future studies are planned to assess the validity of these results in primary B-cells treated *in vitro*

with TCDD and LPS, as well as to confirm the correlation between changes in methylation status with alterations in mRNA expression of specific genes.

# 3. EVALUATION OF ALTERATIONS IN GENE EXPRESSION IN THOSE GENES EXHIBITING ALTERED DNA METHYLATION IN MURINE SPLENOCYTES EXPOSED *IN VIVO* TO LIPOPOLYSACCHARIDE (LPS) AND 2,3,7,8-TETRACHLORODIBENZO-ρ-DIOXIN (TCDD)

McClure, E.A., North, C.M., Kaminski, N., and Goodman, J.I.

a manuscript in preparation for submission to *Toxicology Letters*, 2010

## 3.1 Abstract

2,3,7,8-tetrachlorodibenzo-ρ-dioxin (TCDD) inhibits lipopolysaccharide (LPS)-stimulated differentiation of B-cells within the splenocyte population into antibody-producing plasma cells. We hypothesize that altered DNA methylation, an epigenetic event, plays a key role in this inhibition. DNA was isolated from splenocytes prepared 6 days post experiment initiation from 5-6wk old female C57BL/6 mice dosed with: TCDD, 30μg/kg, on day 0; or LPS, 25μg/mouse, on day 4; or with TCDD and then LPS on days 0 and 4, respectively. Regions of altered DNA methylation (RAMs) were discerned previously by an arbitrarily primed PCR/capillary electrophoresis procedure (Bachman et al., 2006; McClure et al., 2010). The mRNA expression of selected genes (annotated RAMs or genes closely affected by them) that might affect B-cell differentiation were analyzed using qRT-PCR. Three patterns were observed: no change, similar changes in all groups, and different changes based upon treatment. Adcy5 (calcium-dependent adenylyl cyclase) and Bank1 (B-cell protein involved in calcium mobilization) increased mRNA expression in the LPS group and decreased mRNA expression in the TCDD and TCDD+LPS groups. Bcor (transcriptional regulator, can direct epigenetic modifications) decreased mRNA expression in the LPS group, did not change mRNA expression in the TCDD or TCDD+LPS groups.

Ddx54 (repressor of nuclear receptor transcriptional activity), Ralgds (affects ras signaling), Ube2l6 (ubiquitinates proteins) and Phlpp (inhibits Akt phosphorylation) decreased mRNA expression in the TCDD and TCDD+LPS groups, did not change mRNA expression in the LPS group. This study suggests that TCDD blocks LPS-induced B-cell differentiation by affecting both methylation status and gene expression. Further, genes likely important to LPS signaling and TCDD response as well as many LPS signaling genes that are affected by exposure to TCDD are identified.

## 3.2 Introduction

TCDD is a widespread environmental contaminant with high lipophilicity, accumulating in the fat of carnivorous animals (including humans). Specific immunologic aberrations observed in mice exposed to TCDD include the inability to mount an innate immune response to LPS challenge.

LPS is a bacterial endotoxin found in the outer membrane of gram-negative bacteria and is a potent B-cell mitogen capable of stimulating the humoral immune response. Upon stimulation, B-cells become activated and begin rapidly proliferating concomitant with increased expression of surface and secreted immunoglobulin M (IgM).

Murine TCDD-induced immunological suppression is easily measured as a suppression of IgM production with maximum *in vitro* suppression of primary sheep erythrocyte IgM response observed at 30$\mu$g/kg TCDD in splenocytes isolated from C57BL/6 mice (Figure 2.1) (Vecchi et al., 1980). The immune suppression in splenocytes treated *in vivo* with TCDD+LPS is also accompanied with decreased B-lymphocyte-induced maturation protein 1 (Blimp1) expression, decreased $CD19^{+}$ cells, decreased expression of immuno-

globulin J (IgJ), κ, and $\mu$ chains, and decreased major histocompatability complex (MHC) class II expression (North et al., 2009).

Genomic effects of TCDD are mediated almost entirely through binding to the ligand dependent transcription factor aryl-hydrocarbon receptor (AhR). The AhR is normally found in the cytoplasm of cells in complex with two heat shock protein 90kDa (Hsp90), aryl-hydrocarbon receptor interacting protein (AIP), and a heat shock protein co-chaperone p23/prostaglandin E synthase 3 (p23). Upon TCDD binding, AhR releases all chaperone proteins and translocates to the nucleus where it is bound by aryl-hydrocarbon nuclear translocator (ARNT) and functions as a transcriptional regulator which binds dioxin response element (DRE)s (5'-TNGCGTG-3). The ARNT/AhR complex upregulates transcription of NF-E2 related factor 2 (Nrf2), which inhibits nuclear factor κ-light-chain-enhancer of activated B-cells (NFκB) activation. Hsp90 is also a chaperone for inhibitor of nuclear factor of κ light chain gene enhancer in B-cells (IκB) kinase (IKK), which inhibits IKK from inhibiting NFκB (so activating NFκB).

NFκB is composed of heterodimers of subunits p50, RelA, p52, c-Rel, and RelB. Vogel and Matsumura (2009) have identified a RelB/AhR response element (RelBAhRE) which binds a RelB/AhR dimer complex and induces transcription in a TCDD-mediated manner. Binding of this dimer to RelBAhRE's induces transcription of immunologically important genes, including interleukin-8 (IL-8) (Vogel and Matsumura, 2009). The RelB/AhR dimer is also capable of binding DREs, and NFκB binding sites. In the absence of exogenous ligands (TCDD), RelB/p52 binding sites are targeted by RelB/AhR complexes and induce normal trasncription of genes. Further endogenous activation of AhR is observed as a response to cAMP production.

Upon systemic challenge, LPS is bound by soluble acute phase protein LPS binding protein (LBP). The LPS-LBP complex is then recognized and bound by the transmem-

brane CD14 receptor to interact with the lymphocyte antigen 96 (MD2)/toll-like receptor 4 (TLR4) complex. Binding of TLR4 results in its cytoplasmic toll/interleukin-1 receptor (TIR) domain activation. TLR4's TIR domain activates two separate pathways: 1) The Myeloid differentiation primary response gene 88 (MyD88) recruits interleukin-1 (IL-1) receptor associated kinase (IRAK) for phosphorylation and subsequent association with tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6), leading to activation of two signaling pathways. These signaling pathways result in the activation of transcription factors NFκB and activator protein 1 (AP-1). 2) TIR domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) activate TRAF6 which then activates TANK-binding Kinase 1 (TBK1) to phosphorylate transcription factor interferon regulatory factor 4 (IRF4) (Takeda and Akira, 2004). Hsp90 is also (in high concentration) reported to induce TLR4 activation and positively contribute to antigen processing and presentation on MHC class II molecules (Tobian et al., 2004).

TLR4 and B-cell receptor (BCR) signaling also induce Bruton's tyrosine kinase (Btk) to bind phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$) and phosphorylate phospholipase C (PLC). Activated PLC hydrolyzes phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) to inositol triphosphate ($IP_3$) and diacylglycerol (DAG) which regulate further signaling cascades in the cell (ie. calcium release). $PIP_3$ is generated by phosphoinositide 3-kinase ($PI_3K$) (regulated by Lyn of BCR signaling) and recruits Akt to the membrane for activation, with $PIP_3$ binding to Akt identified as the rate-limiting step in Akt activation. Akt signaling is important in regulating survival, cell cycle, metabolism, and apoptosis through regulation of Ras/Raf, mitogen-activated protein kinase (MAPK), and NFκB signaling cascades.

IRF4 is positively regulated by NFκB and indirectly increases Blimp1 expression by inhibiting B-cell lymphoma 6 protein (Bcl6) and paired box protein 5 (Pax5) expression. Together, Bcl6, Blimp1, and Pax5 create a reciprocally repressing transcription factor switch, with Pax5 inhibiting IgM production (Figure 1.1).

Epigenetic mechanisms of regulation occur through the addition of heritable, impermanent marks upon genetic material. Epigenetic regulation can be passed from parental to filial generations of both cells and whole organisms. More transient occurrences are also identified in which various stages of cellular development and differentiation are regulated epigenetically.

DNA methylation, in the form of 5-methylcytosine, normally occurs at 70% of CpG dinucleotides (Naveh-Many and Cedar, 1981b). Altered DNA-methylation, an epigenetic mechanism that plays a regulatory role in gene expression, has been proven to play multiple roles in carcinogenesis, development, differentiation and even TCDD response (Kurkjian et al., 2008; Okino et al., 2006; Wu et al., 2004).

A change in DNA methylation due to LPS-challenge *in vivo* has previously been described as have alterations in DNA methylation due to TCDD exposure alone and prior to LPS-challenge (McClure et al., 2010). In this letter, evidence is presented that suggests some genes associated with regions of altered DNA methylation (RAMs) also exhibit changes in mRNA expression due to LPS and TCDD exposure.

## 3.3 Materials and Methods

### 3.3.1 Preparation of *in vivo* Splenocyte Samples

#### Chemicals

TCDD was purchased from Accustandard (New Haven, CT) and prepared in sesame oil (Sigma-Aldrich, St. Loius, MO). *Salmonella typhosa* LPS (Sigma-Aldrich, St. Louis, MO) was prepared in phosphate buffered saline (PBS) immediately prior to administration.

#### Animals

Mice, treatments, and splenocyte collection were described previously (North et al., 2009). The same splenocyte samples from those animals sacrificed upon day 6 (post LPS exposure) in the previous studies (North et al., 2009; McClure et al., 2010) were used for this study. Female 6-8 week old C57BL/6 mice were purchased from the National Cancer Institute and housed in accordance with Michigan State University Institutional Animal Care & Use Committee policy. On day 0, TCDD (0, 3, 10, or 30$\mu$g/kg in sesame oil) was administered by single oral gavage. On Day 4, to initiate primary humoral immune response, LPS (0 or 25$\mu$g in PBS) was administered by intraperitoneal injection. Spleen samples were collected on days 4-7 from all treatment groups (6 animals per group) and stored at -80$^{\circ}$C. Mice from day 4 that received only TCDD or vehicle treatment, were used to establish baseline TCDD effects (measured by Antigen Forming Colonies (AFCs) response from samples collected on day 7) (Figure 2.1).

### 3.3.2 qRT-PCR

#### RNA Isolation

RNA from splenocytes was isolated using TRIzol® Reagent (Sigma-Aldrich, St. Louis, MO) according to manufacturer's protocol. Following isopropanol precipitation and ethanol wash, RNA pellets were resuspended in Promega SV RNA Lysis Solution and further purified according to the manufacturer's protocol (Promega, Madison, WI).

#### cDNA Generation

cDNA was generated using Applied Biosystems High Capacity Archive kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA).

#### Gene Selection Criteria

Gene selection criteria is based upon data published in McClure et al. (2010). Each gene examined via quantitative reverse transcription PCR (qRT-PCR) was chosen for unique reasons as listed below:

- *Adenylate cyclase type 5 (Adcy5)* is activated by calcium signaling.
- In the previous study, *protein kinase B (Akt)* was uniquely identified as a common target of annotated genes occurring in groups treated with 25$\mu$g LPS, 30$\mu$g/kg TCDD, or 30$\mu$g/kg TCDD+LPS.
- Few studies have reported a change in Akt expression as a regulatory mechanism in cell development, while many report changes in the phosphorylation state (and so activity) of this kinase. For this reason, the expression of *PH domain and leucine*

*rich repeat protein phosphatase (Phlpp)*, a dephosphorylase uniquely identified as specifically targeting Akt, was assessed.

- *B-cell scaffold protein with ankyrin repeats (Bank1)* was selected due to its ability to regulate calcium signaling specifically in activated B-cells.

- *Bcl6 co-repressor (Bcor)* functions in concert with Bcl6 in 'switch' regulatory sequence and was cloned from treatments although never annotated (unpublished data).

- *Calcium-dependent secretion activator 2 (Cadps2)* participates in the priming step of dense-core vesicle exocytosis, an important function in various calcium-secreting cells (Sadakata et al., 2007). Study of mouse tissue homogenates indicates that Cadps2 is most highly expressed in the brain, pituitary, and lungs but only minimally expressed in the spleen and thymus (Sadakata et al., 2007). The pleckstrin homology domain of Cadps2 likely allows interaction with $PIP_2$-rich microdomains in the plasma membrane in a calcium-dependent manner. Although it has not yet been studied nor proven, it is possible that Cadps2 helps in vesicle-mediated immunoglobulin secretion in differentiated B-cells.

- *Dead box 54 (Ddx54)* was annotated in all treatments, but identified as having targets common with other annotated genes only in LPS and 30$\mu$g/kg TCDD and so was assessed as an anomalous representative of the annotated gene populations.

- *Interleukin-17 receptor D (Il17rd)* affects Ras, MAPK, and Akt.

- *Small subunit processome component homolog (Krr1)* is a ribosomal protein down-regulated in metastatic histiocytoma (Adrien et al., 2010).

- *Tyrosine-protein phosphatase non-receptor type 3 (Ptpn3)* is affected by ubiquitin protein ligase E3A (Ube3A) and inhibiting MAPK.

- *Ral guanine nucleotide dissociation stimulator (Ralgds)* is important in G-Protein Coupled Receptor (GPCR) signaling.

- *Retinoic acid receptor α (Rarα)* is a nuclear receptor which increases T-lymphocyte number, NO synthase activation, and LBP expression (Seguin-Devaux et al., 2002, 2005).

- *Ubiquitin/ISG15-conjugating enzyme E2 L6 (Ube2l6)* functions in concert with Ube3A to affect Akt activity.

- *Zinc finger protein 128 (Zfp128)* is a poorly studied zinc-finger protein with likely transcription factor activity.

These genes have all been identified as potentially important in B-cell development, differentiation, and/or signaling.

**Primer Preparation**

Primers were designed using the web-based NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were synthesized by the Macromolecular Structure Facility at Michigan State University. The UCSC In-Silico PCR web-based tool (July 2007 build, http://genome.ucsc.edu/cgi-bin/hgPcr?db=mm9) confirmed designed primers to preclude the possibility that expression data be attributed to genomic DNA contamination. Through designing primers to span an exon-exon junction and so that products span at least one intron we have increased stringency of primer selection to ensure

evaluation of changes in functional mRNA expression. Gene names, symbols, accession numbers, primer sequences, and amplicon size are listed in Table S.1.

**mRNA Quantification**

According to the manufacturer's protocol, each reaction contained 1$\mu$L of cDNA from the aforementioned reverse transcription reaction (with the exception of 18s reactions, which contained 1$\mu$L of 1:1000 cDNA in DEPC-treated water), 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.3$\mu$M of forward and reverse primers, and DEPC-treated water (Ambion, Austin, TX) to 50$\mu$L. QRT-PCR amplification of duplicate reactions was conducted as previously described (Phillips et al., 2009). Using the absolute quantitation method of determining mRNA expression levels, mRNA copy number of genes was standardized to that of the 18S rRNA gene copy number to control for differences in RNA quantity, quality, and reverse transcription efficiency between samples.

**Data Analysis**

Fold changes in the treatment groups (vs. control) were calculated by comparing: (1) 30$\mu$g/kg TCDD+LPS versus Control, (2) 30$\mu$g/kg TCDD versus Control, (3) LPS versus Control, (4) 3$\mu$g/kg TCDD+LPS versus Control, and (5) 3$\mu$g/kg TCDD versus Control. Statistical outliers, identified by the Grubbs's test ($p \leq 0.05$, http://www.graphpad.com/quickcalcs/Grubbs1.cfm) were excluded from the final fold change calculations. Expression was considered differentially regulated if it was statistically different from the control group as determined by Students two-tailed t-test, $p \leq 0.05$. Cases in which there was no statistically significant change, but gene expression in $\geq 3$ samples was outside of the 95% confidence interval (CI) of the control group, and all in the

same direction (either up or down), were considered to show an "indication of change" in expression. Results are presented in Tables 3.1 and S.4.

## 3.4 Results and Discussion

qRT-PCR analysis assessed mRNA expression of 14 genes (see section 3.3.2 for a description of selection criteria) which can affect crucial pathways involved in the differentiation and proliferation of B lymphocytes (Figures 3.1 and 3.2): 7 annotated genes plus 6 genes which interact with Akt-PI3K regulation of B-cell maturation plus Akt. Eleven of these genes exhibit altered expression in at least one treatment group, while the other 3 show an indication of altered expression (Figure 3.1, Tables 3.1 and S.4).

The only gene to show no change in expression due to treatment, Il17rd mRNA expression is decreased in splenocytes of mice treated with 30$\mu$g/kg TCDD, 25$\mu$g LPS, or 30$\mu$g/kg TCDD+LPS (Tables 3.1 and S.4). Decreased Il17rd mRNA expression may decrease its inhibition of Akt, increasing cAMP response element-binding protein (CREB), estrogen receptor (EsR), mammalian target of rapamycin (mTOR), IKK, and glycogen synthase kinase 3$\beta$ (GSK-3$\beta$) activities. In concert, these changes increase Myc and Blimp1 transcription, decreasing Pax5 activity and increasing IgM expression (Figure 3.2). Il17rd is newly methylated in an intron in splenocytes of animals treated with 30$\mu$g/kg TCDD+LPS (McClure et al., 2010). The methylation status of this gene does not correlate with the decreased expression in all treatment groups. Because the decrease in Il17rd mRNA expression is observed as a result of all three treatments, likely this downregulation is not the main regulatory mechanism by which TCDD inhibits the differentiation of plasma cells in response to LPS stimulation.

Table 3.1: **Treatment-related changes in gene expression.**

| Gene[a] Symbol | Expression Change[b] | | | Gene Description/Function | NCBI RefSeq |
|---|---|---|---|---|---|
| | LPS | 30μg/kg TCDD | 30μg/kg TCDD+LPS | | |
| Akt | ↑ | – | – | Serine/Threonine Kinase Capable of regulating proliferation | NM_009652 |
| Adcy5 | ↑ | ↓ | ↓ | $Ca^{2+}$ dependent Adenylcyclase | NM_001012765 |
| **Bank1** | – | ↓ | ↓ | B-Cell adaptor protein capable of inhibiting Akt activation | NM_001033350 |
| **Ddx54** | – | ↓ | ↓ | RNA Helicase able to repress nuclear receptor transcriptional activity | NM_028041 |
| Phlpp | – | ↓ | ↓ | Inhibitor of Akt phosphorylation and activity | NM_133821 |
| **Ralgds** | – | ↓ | ↓ | Stimulates dissociation of GDP from Ras-related GTPases | NM_009058 |
| Ube2l6 | – | ↓ | ↓ | Ubiquitin-Conjugating Enzyme | NM_019949 |
| Bcor | ↓ | – | – | Bcl6 Co-Repressor, transcriptional regulator May Affect Epigenetic Modifications | NM_175045 |
| **Zfp128** | ↓ | – | – | Represses BMP/TGF-β signaling through SMAD1 | NM_153802 |
| **Il17rd** | ↓ | ↓ | ↓ | Inhibits FGF signaling (and so proliferation) through the FGFR | NM_134437 |
| Ptpn3 | ↓ | – | ↓ | Protein Tyrosine Phosphatase, Non-Receptor Type 3 | NM_011207 |

[a] Analyzed genes include 5 annotated genes (**bold**) and 6 other genes (selection criteria: see section 3.3.2), detailed data presented in Table S.4

[b] All expression changes are statistically significant (as measured by Student's, two-tailed, t-test, $p \leq 0.05$) and indicated as: upregulation (↑), downregulation (↓), or no change (–)

### 3.4.1 Genes Involved in LPS Signaling

Ptpn3 mRNA expression is not altered in splenocytes of mice treated with 30μg/kg TCDD, but decreased in those treated with 30μg/kg TCDD+LPS or 25μg LPS (Tables 3.1 and S.4). Cadps2 mRNA exhibits indication of a similar pattern of expression (Tables S.4). Decreased Ptpn3 mRNA may increase MAPK signaling and so AP-1 activity. Increased AP-1 activity increases Blimp1 transcription, so decreasing Pax5 activity and increasing IgM expression (Figure 3.2). Decreased Cadps2 mRNA may be also be an important component of LPS signaling that results in IgM secretion, that is not affected by TCDD exposure.

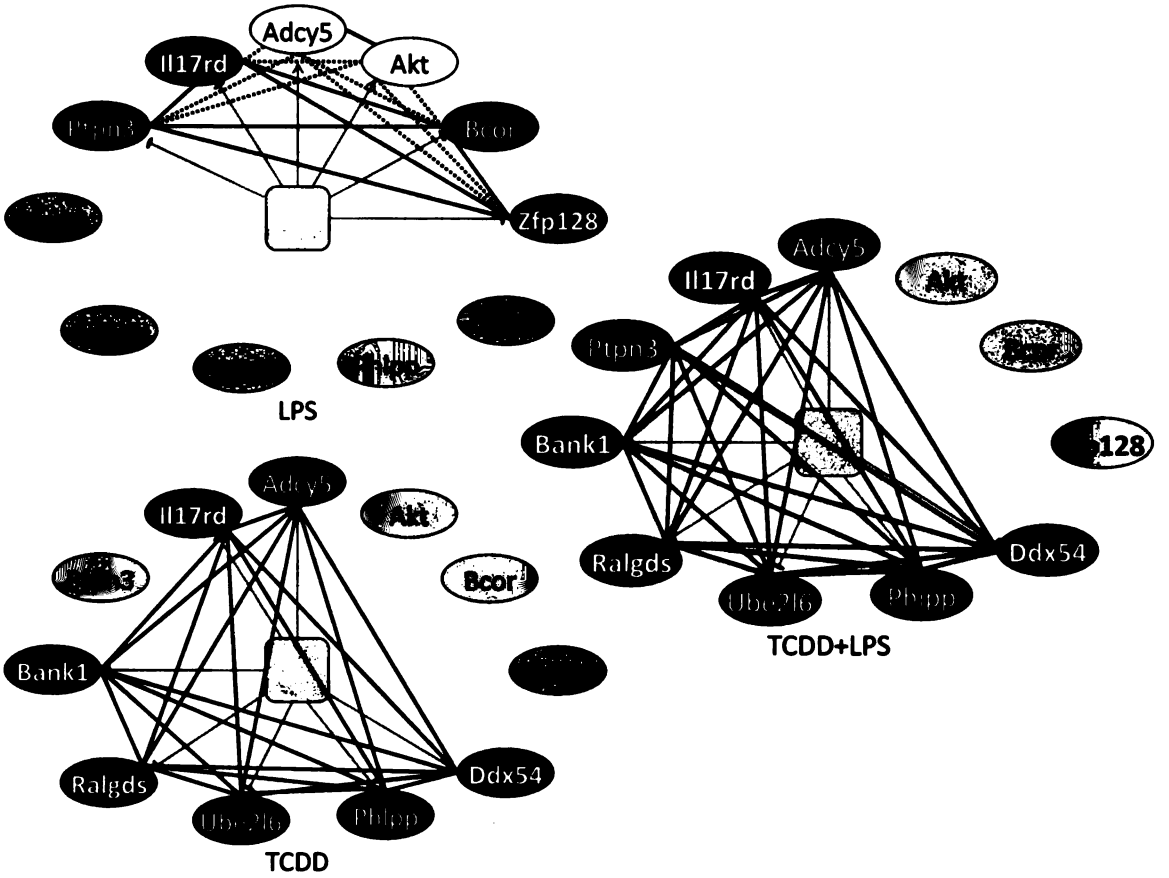



Figure 3.1.: **Treatment-related changes in splenocyte gene expression.** Expression of 5 annotated **(bold)** and 6 additional genes (selection criteria presented in section 3.3.2) was evaluated (upregulated: white, →; downregulated: black, ⊣; or unchanged: grey) following treatment with 25$\mu$g LPS, 30$\mu$g/kg TCDD, or 30$\mu$g/kg TCDD+LPS. Genes exhibiting expression changes in the same direction (either up or down) are connected with a solid line, those exhibiting expression changes in opposite directions are connected with a dashed line.

Cadps2 annotates to a new methylation, ≤2kb downstream of the gene, unique to 30$\mu$g/kg TCDD+LPS treatment and occurs within 2kb of the last exon/intron of the gene (McClure et al., 2010). Because the region of altered DNA methylation (RAM) annotated to Cadps2 is unique, it was not also observed in groups treated with LPS indicating that this change in DNA methylation is not specific to LPS signaling (like the gene expression data) and so cannot be correlated with gene expression.

Because Ptpn3 and Cadps2 expression are decreased only in the splenocytes of those animals treated with LPS, it is likely that these proteins help regulate IgM expression in response to LPS challenge. However, IgM expression, but neither Ptpn3 nor Cadps2 expression, is greatly diminished in those splenocytes of mice exposed to TCDD prior to LPS challenge (North et al., 2009), likely indicating that the downstream effects of Ptpn3 expression are also altered by TCDD exposure.

### 3.4.2 Genes Involved in TCDD Signaling

Ddx54, Ralgds, Bank1, Phlpp, and Ube2l6 mRNA expression are not altered in splenocytes of mice treated with 25$\mu$g LPS but decreased in those treated with 30$\mu$g/kg TCDD or 30$\mu$g/kg TCDD+LPS (Tables 3.1 and S.4). Decreased Ddx54 mRNA may decrease its inhibition of EsR and so increase the transcription of genes regulated by EsR, including those with enhanced expression when AhR and EsR concomitantly bind the promoter region. Ddx54 also has helicase activity and so may regulate other genes epigenetically, an action that may be inhibited by Ddx54 downregulation (Figure 3.2). Decreased Ralgds mRNA may decrease 3-phosphoinositide dependent protein kinase-1 (PDK1) and AP-1 activity. In concert, these changes decrease Blimp1 transcription, increasing Pax5 activity and decreasing IgM expression. Decreased Bank1 mRNA may decrease release of calcium from internal stores, reducing calmodulin (CaM) inhibition of E2A (critical to promoting the expression of pro-B-cell proteins such as recombination activating gene (RAG)), increasing v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets1) and EsR suppression, reducing NFκB and IRF4 inhibition, and eventually activating Blimp1 which results in inhibited Pax5 and increased IgM expression. Decreased Phlpp mRNA may decrease its inhibition of Akt, increasing CREB, EsR, mTOR, IKK, and GSK-3β activity. Decreased

Ube2l6 mRNA may increase Ptpn3s inhibition of MAPK and decrease Ube3As activation of Akt, decreasing AP-1 activity while increasing CREB, EsR, mTOR, IKK, and GSK-3β activity (Figure 3.2).

Rarα mRNA expression is not altered in splenocytes of mice treated with 25μg LPS but shows indication of increased expression in those treated with 30μg/kg TCDD or 30μg/kg TCDD+LPS (Tables S.4). An increase in Rarα mRNA may increase LBP expression (and so LPS binding and recognition by lymphocytes) as well as NO synthase activity (a process also induced by LPS signaling through TLR4). Oxidants impede Nrf2 degradation, increasing its translocation to the nucleus, and likely reducing NFκB-mediated pro-inflammatory reactions through competition for binding to aryl-hydrocarbon response elements (AREs) (Li et al., 2008). TCDD exposure induces Nrf2 transcription, thereby causing reduced cellular LPS-induced inflammatory response, a mechanism likely developed to inhibit hypersensitive response to pathogens (Thimmulappa et al., 2006) (Figure 3.2). However, when induced by TCDD exposure prior to LPS challenge the reduction in inflammatory response induced by Nrf2 signaling may be exaggerated to such an extent that instead of protecting against LPS-induced septic shock, it actually inhibits response to the challenge and so limits appropriate B-cell differentiation. The upregulation of Rarα in 30μg/kg TCDD and 30μg/kg TCDD+LPS increases both the recognition of LPS and the translocation of Nrf2 to the nucleus to inhibit inflammatory response. These actions work in opposition, however, because that reducing inflammatory response acts in *cis* to affect the originating cell while that increasing LBP expression acts in *trans* to affect other cells as well as the originating, it is likely that the former mechanism gains precedence and serves as a means by which TCDD exposure inhibits the differentiation of B-cells in response to LPS challenge.

Ralgds, Bank1, and Phlpp did not annotate to RAMs in any treatments. Ddx54 is hypomethylated at a location ≥2kb upstream of the transcriptional start site (TSS) in 25μg LPS, 30μg/kg TCDD, and 30μg/kg TCDD+LPS treatment groups. Ube2l6 is hypermethylated at a location ≥2kb upstream of the TSS in 30μg/kg TCDD+LPS (McClure et al., 2010). This data suggests that the alterations in gene regulation observed as a result of TCDD treatment were not dependent upon methylation status in the examined regions.

In concert, decreases in Ddx54, Ralgds, and Ube2l6 mRNA and increases in Rarα expression result in increased EsR activity, increased Nrf2 activity, decreased Blimp1 activity, and decreased IgM expression: a pattern observed in splenocytes of mice treated with 30μg/kg TCDD or 30μg/kg TCDD+LPS. Decreases in Bank1 and Phlpp mRNA expression are hypothesized to decrease EsR activity, decrease release of calcium from internal stores, increase Blimp1 activity, and increase IgM expression. However, decreased Blimp1 may also function by decreasing inositol triphosphate receptor ($IP_3R$) mediated $Ca^{2+}$ release from the endoplasmic reticulum, resulting in decreased protein kinase C (PKC) activity, leading to decreased ras/raf signaling, decreased MAPK signaling, decreased AP-1 activity, which can downregulate Blimp1, activating Pax5 and decreasing levels of IgM mRNA. The described pattern (as well as that hypothesized to occur as a result of decreases in Ddx54, Ralgds, and Ube2l6 mRNA expression) of gene downregulation in 30μg/kg TCDD and 30μg/kg TCDD+LPS treatments is consistent with the observation that IgM expression is suppressed as a result of TCDD exposure. This result indicates that Rarα, Ddx54, Ralgds, Bank1, and Ube2l6 mRNA downregulation may be important signaling events occurring in TCDD-exposed splenocytes.

### 3.4.3 Genes Involved in LPS Signaling and Affected by TCDD exposure

Akt mRNA expression is not altered in splenocytes of mice treated with 30$\mu$g/kg TCDD nor 30$\mu$g/kg TCDD+LPS but is increased in those treated with 25$\mu$g LPS. Bcor andZfp128 mRNA is not altered in splenocytes of mice treated with 30$\mu$g/kg TCDD nor 30$\mu$g/kg TCDD+LPS but decreased in those treated with 25$\mu$g LPS

(Tables 3.1 and S.4). Increased Akt mRNA may increase the concentration of active Akt and so CREB, EsR, mTOR, IKK, and GSK-3$\beta$ activity. Decreased Bcor mRNA may decrease the activity of Bcl6, thereby increasing Blimp1 and decreasing the activity of Pax5 and B-cell lymphoma-extra large (Bcl-xl), so increasing IgM expression and apoptosis (Figure 3.2).

Krr1 mRNA expression is not altered in splenocytes of mice treated with 30$\mu$g/kg TCDD nor 30$\mu$g/kg TCDD+LPS but shows indication of decreased expression in those treated with 25$\mu$g LPS (Table S.4). This decrease in expression is correlated with hyper-methylation of the gene's TSS (McClure et al., 2010) and suggests that LPS signaling induces methylation of Krr1's TSS in response to LPS-challenge in order to decrease mRNA expression. Because Krr1 is a subunit of a processome important in ribosomal biogenesis, it is likely that downregulation of this gene's expression may have global impact in inhibiting cellular transcription and translation.

In concert, these changes increase Myc and Blimp1 transcription, decreasing Pax5 activity and increasing IgM expression (Figure 3.2). Because the hypothesized effects of the observed increase in Akt and Bcor expression are in accordance with those reported by North et al. (2009), it is reasonable to hypothesize that these gene expression alterations may be responsible for the increased IgM expression induced by LPS-challenge. The

lack of gene expression in those splenocytes of animals treated with 30μg/kg TCDD+LPS indicates that TCDD exposure inhibits appropriate gene regulation for LPS-signaling.

Adcy5 mRNA expression is increased in splenocytes of mice treated with 25μg LPS but decreased in those treated with 30μg/kg TCDD, or 30μg/kg TCDD+LPS (Tables 3.1 and S.4). Decreased Adcy5 mRNA may decrease renin (REN) activity, acting upon downstream elements to decrease Blimp1 transcription, increasing Pax5 activity and decreasing IgM expression. Altered Bank1 mRNA may affect calcium ($Ca^{2+}$) release from internal stores, acting upon downstream elements to affect Blimp1 transcription, affecting Pax5 activity and IgM expression (Figure 3.2). Because Adcy5 expression is decreased only in the splenocytes of those animals treated with TCDD and is increased in those treated with only LPS, it is likely that this protein, as well as Akt, regulates pathways important in LPS response and disrupted in response to TCDD exposure.

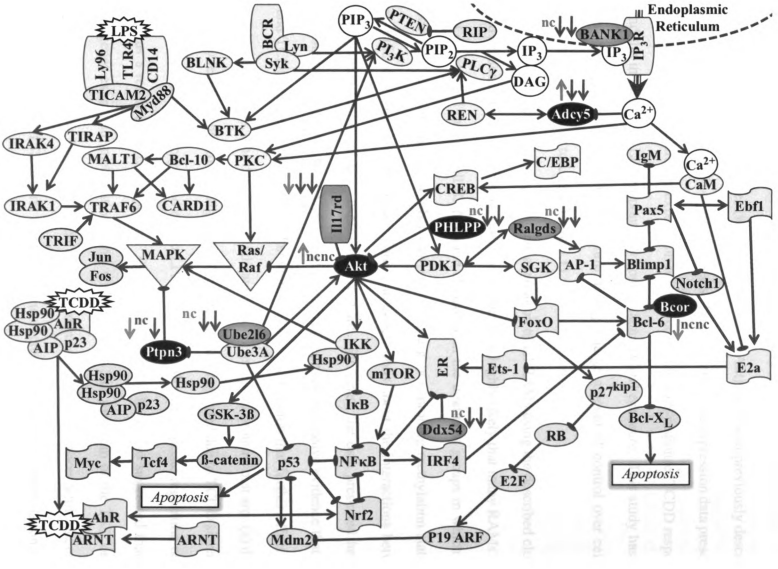
The similar pattern in mRNA expression of numerous genes, with similar regulation in TCDD and TCDD+LPS treatments but dissimilar regulation in LPS treatment, indicates that TCDD has a dominant influence upon the mRNA expression of these genes. Whether this influence is through many different affects of TCDD or simple alterations in the expression of specific transcription factors is yet to be determined, although the de-regulation of Blimp1 expression is a likely candidate as it has been shown to be a master-regulator in the development of B cells and is a transcription factor capable of regulating many signaling pathways within the cell.

Bank1, Ddx54, Phlpp, Ralgds, and Ube2l6 are all downregulated in 30μg/kg TCDD and 30μg/kg TCDD+LPS treatments, but not in LPS treatment. Ptpn3 is downregulated in LPS and 30μg/kg TCDD+LPS treatments but not in 30μg/kg TCDD treatment. This evidence would indicate that the regulation of mRNA expression of these genes is only by TCDD or LPS treatment, whether alone or in combination. Unfortunately, due to limitations in

the sequencing protocol, direct comparison of changes in methylation status to mRNA expression cannot be made. Future work including bisulfite sequencing of areas of interest in specified genes will be better able to elucidate these connections.

Figure 3.2. : **Hypothesized significance of interactions occurring in differentiating splenocytes.** (This image is presented in color). Expression of 5 annotated (orange) and 5 additional genes (black) (selection criteria presented in section 3.3.2) was analyzed from splenocytes treated *in vivo* with 25$\mu$g LPS (red), 30$\mu$g/kg TCDD (green), and 30$\mu$g/kg TCDD+LPS (blue) treatments. Changes in mRNA expression are indicated next to genes as an increase ($\uparrow$), decrease ($\downarrow$), or no change (nc). Positive regulation ($\rightarrow$) and negative regulation ($\dashv$) of genes are indicated.

Bank1 was upregulated following LPS treatment. This is hypothesized to increase $IP_3R$ mediated $Ca^{2+}$ release from the endoplasmic reticulum, resulting in increased PKC activity, leading to increased ras/raf signaling, increased MAPK signaling, increased AP-1 activity, which can up-regulate Blimp1, inhibiting Pax5, which is expected to increase IgM levels. By similar logic, Bank1 is downregulated in 30$\mu$g/kg TCDD treatment, which is anticipated to decrease IgM levels.


LPS
Ly96
TLR4
CD14
TICAM2
Myd88
BCR
Lyn
Syk
BLNK
BTK
IRAK4
TIRAP
IRAK1
TRAF6
TRIF
MALT1
Bcl-10
CARD11
PKC
MAPK
Jun
Fos
Ras/
Raf
Akt
PIP3
PTEN
PI3K
PIP2
RIP
PLCγ
IP3
DAG
BANK1
IP3R
Endoplasmic
Reticulum
REN
Adcy5
Ca2+
IgM
CaM
C/EBP
CREB
PHLPP
Ralgds
PDK1
SGK
AP-1
Pax5
Ebf1
Blimp1
Notch1
Bcor
Bcl-6
FoxO
E2a
Ets-1
ER
mTOR
IKK
IκB
NFκB
p53
IRF4
Ddx54
p27kip1
Bcl-XL
RB
E2F
Apoptosis
Myc
Tcf4
β-catenin
GSK-3β
Ptpn3
Ube2l6
Ube3A
Hsp90
AIP
p23
AhR
ARNT
TCDD
Nrf2
Mdm2
P19 ARF
IL17rd
nc
ncnc

### 3.4.4 Conclusion

This analysis has identified three genes involved in TCDD signaling and eight genes involved in LPS signaling, of which six are adversely affected by concomitant TCDD-exposure. None of these genes (with the exception of Akt) have been previously described as being involved in TCDD or LPS responses. Taken together, the expression data presented here describes novel protein expression patterns critical to LPS response, TCDD response, and the inhibition of LPS response by concomitant TCDD exposure. This study has also described evidence for TCDD-exposure having a large amount of control over cellular responses, especially to LPS (Figure 3.1).

As discussed previously, complete annotation of all RAMs using the described cloning and sequencing technique is not possible. In fact, it is highly likely that many RAMs were not cloned and that not all RAMs were cloned in all the treatment groups in which they occurred. For this reason, further analysis of these genes, probing methylation status at specific sites must be employed before final conclusions about the interactions between methylation status and gene expression are made. However, the data presented here in combination with that from McClure et al. (2010) supplies persuasive evidence that LPS and TCDD exposure cause novel alterations in DNA methylation that further effect gene mRNA expression. The data further indicates 3 novel groups of proteins that are: (a) differentially regulated due to LPS signaling, (b) differentially regulated due to TCDD signaling, and (c) differentially regulated due to LPS signaling, with inappropriate regulation occurring as a result of TCDD exposure. Results of this study have also suggested that, for the genes examined, methylation status within introns, 2kb upstream of the TSS, or 2kb downstream of the last exon/intron does not regulate gene expression while increased methylation in the TSS of Krr1 decreases gene expression. The DNA methylation status

and gene expression data for Krr1 indicate that TCDD exposure has affected both. This discovery suggests a novel epigenetic mechanism of TCDD-induced gene regulation by which TCDD affects gene expression through methylation of the TSS. This discovery is contrary to the previously held opinion that alterations in gene expression induced by TCDD exposure are dependent upon genetic mechanisms involving the binding of TCDD-bound AhR to gene promoter elements. The evidence of an epigenetic mechanism of regulation occurring within splenocytes exposed to TCDD suggests the occurrence of more complicated interactions that were previously hypothesized.

# 4. ADDITIONAL RESULTS

In addition to those results discussed in chapters 2-3, indications of DNA methylation affecting genes important for other epigenetic mechanisms of regulation have been discovered:

**miRNA** One RAM annotated to a mouse microRNA, mmu_miR_210. mmu_miR_210 is a key player of endothelial cell response to low oxygen tension and is induced by HIF1 (Fasanaro et al., 2008). In humans, *in silico* searches have revealed that genes involved in proliferation, DNA repair, chromatin remodeling, metabolism, and cell migration (specifically E2F transcription factors and Acvr1b) may all be targets of miR_210 (Fasanaro et al., 2008) (Kulshreshtha et al., 2008).

Two genes important in B-cell differentiation and survival, c-Myc and E2F, activate the miR_17_92 oncogenic cluster (Ivan et al., 2008) while tumor protein 53 (p53) targets miR_34a and contributes to its function (He et al., 2007). Together, these interactions suggest a role for miRNA in development of splenocytes and their signaling functions.

**Repeat Elements** A total of 29 annotated genes and 34% of RAMs not annotated to genes contain long interspersed nuclear element (LINE) elements. Because CpG dinucleotide methylation protects healthy cells fom inappropriate transcription of repetitive elements such as LINEs it is natural to hypothesize that these annotated genes and regions of the chromosome are critically regulated by DNA methylation (Walsh et al., 1998). Future studies should address the methylation status of these regions in B-cells at different stages of development in order to discern if they contain previously unrecognized regulatory elements.

**Transcription Factors** Three annotated genes, E2F8, Taf4a, and Tcf4, are transcription factors. Often transcription factors are regulated in a developmental or tissue-specific manner. Disregulation of the appropriate expression of transcription factors results in inappropriate gene regulation. Future studies should address the expression of these transcription factors in splenocytes as well as throughout B-cell differentiation.

**Histone Code** Two genes, Jarid1a and Taf5l, are capable of altering the histone code. Jarid1a demethylates lysine 4 of histone H3, so regulating transcription of the Hox protein during differentiation. Taf5l is a component of the PCAF complex which is able to acetylate histones in a nucleosomal complex. Alterations in the methylation state of these two genes may further epigenetically regulate the expression of other genes by altering the histone code.

# 5. SUMMARY

Epigenetics is a discipline still in its infancy. Only recently has the general public begun to discover the implications of epigenetic research. This field is exciting because of the many levels of intricacy with which it is associated. No epigenetic event can be taken completely out of context from other epigenetic and genetic factors occurring within a cell or organism. Studies in DNA methylation are necessarily affected by occurrences of mutations, histone alterations, ncRNA, and tissue-specific transcription factors within the same populations. For this reason, minute examination of individuals may not result in the drawing of appropriate conclusions about a population. Rather, groups of individuals must be studied and the average occurrence of epigenetic marks measured in an attempt to develop a snapshot of important regulatory mechanisms.

The studies described in this thesis were designed so as to explore a heterogeneous population of splenocytes in order to better understand the alterations in DNA methylation that occur as a result of LPS-challenge and TCDD exposure. Although DNA methylation states may be similar among studied splenocytes, the occurrence of other epigenetic and genetic markers as well as specific cell subtypes in which the methylations occur can all affect the realization of effect upon gene regulation.

A broad, non-biased approach was adopted in order to obtain a pattern of global alterations in methylation. This non-biased approach still had limitations in complete identification of all RAMs in all treatment groups. In order to confirm methylation status of observed gene regions, more specific studies should be performed including bisulfite sequencing or methylation sensitive qRT-PCR.

Despite the limited nature of these studies, 5 important conclusions can be drawn:

1) LPS-challenge results in alterations in DNA methylation

2) TCDD exposure results in alterations in DNA methylation

3) TCDD exposure prior to LPS-challenge results in alterations in DNA methylation not observed in LPS-challenge or TCDD exposure and indicates an epigenetic form of crosstalk occurring between signaling of the two treatments

4) LPS-challenge results in alterations in gene expression

5) TCDD exposure results in alterations in gene expression

The potential regulation of LPS-induced signaling through specific and global changes in DNA methylation has not been addressed before. This result has larger implication in the treatment of bacterial infection, study of B-cell differentiation, and study of commensal/host interactions. Future work should address questions such as: Do bacterial strains alter DNA methylation differently? Does knockout of DNMT's cause an individual to become more or less susceptible to bacterial infection? Can specific genes be targeted by drug therapies to induce an altered immune response and so better prognosis in difficult-to-treat bacterial infections? These are broad and difficult questions that may be addressed through the application of methylation sensitive PCR, bisulfite sequencing, gene expression profiling, and knockout models.

The potential regulation of TCDD-induced signaling through global changes in DNA methylation has previously been addressed (Wu et al., 2004; Shen and Whitlock, 1989) but not as broadly nor in regards to the specific genes discussed here. The results reported here have larger implications in the treatment of TCDD poisoning. Future work should address questions such as: How long do the patterns of altered DNA methylation last in TCDD exposed populations? Does the knockout or induction of any gene attenuate the ability of TCDD to alter DNA methylation? Can TCDD treatment be useful in illnesses or poisonings from other compounds inducing increased DNA methylation? These, like those of LPS, are broad and difficult questions that may be assessed using similar methods.

These results offer insight into highly complex signaling networks occurring in LPS and TCDD exposed splenocytes 3.2. I propose that these networks are partly regulated by epigenetic changes (specifically DNA methylation) in exposed populations. Future work must address specific sites of altered DNA methylation in correlation with changes in gene expression. These studies may then discover new target genes for treatment of bacterial infection and sepsis, for treating TCDD poisoning, or even for treatment of immunologic deficiencies.

# Appendix A: Supplemental Tables

Table S.1: **Primer sequences of 14 selected[a] genes for qRT-PCR analysis of mRNA expression**

| Gene Symbol[b] | Gene Name | NCBI RefSeq | Forward Primer | # bp | Reverse Primer | # bp | Size[c] (bp) |
|---|---|---|---|---|---|---|---|
| 18s | 18s Ribosomal RNA | | CCGAAGCGTTTACTTTGAA | 19 | CCTCTTAATCATGGCCTCA | 19 | |
| Adcy5 | Adenylate cyclase 5 | NM_001012765 | CCTACCTCAAGGAGCACAGC | 20 | GGATTGTGTCCGATGGAGTT | 20 | 124 |
| Akt | Thymoma viral proto-oncogene 1 | NM_009652 | GATTGTGTCTGCCCTGGACT | 20 | CCCGAAGTCCGTTATCTTGA | 20 | 115 |
| **Bank1** | B-cell scaffold protein with ankyrin repeats 1 | NM_001033350 | AACATTGGCAGATGGGAAAG | 20 | TGGTGCACAATGGTCAGTTT | 20 | 130 |
| Bcor | Bcl6 interacting corepressor | NM_175045 | AGGAAACGCAAACTGTCAGG | 20 | AGTGGCGTTGCTTTTTAGGA | 20 | 127 |
| **Cadps2** | $Ca^{2+}$-dependent activator protein for secretion 2 | NM_153163 | AACAAGAGTTTGCCCACCAC | 20 | GCGCAAGTCAGTTGTTTTGT | 20 | 134 |
| **Ddx54** | DEAD (Asp-Glu-Ala-Asp) box polypeptide 54 | NM_028041 | GGAAGAAGAAGCGGTTTGTG | 20 | CCGGTCATCAATTTTCTGCT | 20 | 140 |
| **Il17rd** | Interleukin 17 receptor D | NM_134437 | GTGGATGACAGCAACACCAC | 20 | GACAACCAGAGGCACAGTGA | 20 | 111 |
| **Krr1** | Small subunit (SSU) processome component | NM_178610 | AAGGAAAAGCCAGCTGTGAA | 20 | CCCAGTTTCTTCGTCTTTGC | 20 | 104 |
| Phlpp | PH domain and leu rich repeat protein phosphatase | NM_133821 | GCATTCCAACCCTAGCAGAG | 20 | ATTTCCATCCAGCAGAAACG | 20 | 107 |
| Ptpn3 | Protein tyrosine phosphatase, non-receptor type 3 | NM_011207 | CTATCTTGCCGACAGCCAGT | 20 | TGTAGCAAGATTCGGCTTCC | 20 | 116 |
| **Ralgds** | Ral guanine nucleotide dissociation stimulator | NM_009058 | AAGAAGACGTGGGAAGAGGTC | 21 | AGTGTGGCAAACTTGGAGGT | 20 | 137 |
| Rara | Retinoic acid receptor, alpha | NM_009024 | CAGAGAGCTACACGCTGACG | 20 | TTGAGGAGGGTGATCTGGTC | 20 | 262 |
| Ube2l6 | Ubiquitin-conjugating enzyme E2L 6 | NM_019949 | CTGGAAGCCTTACACCAAGC | 20 | TCTGAGTCAGGAGGTCAGCA | 20 | 113 |
| **Zfp128** | Zinc finger protein 128 | NM_153802 | ACTTTCCGAGATGTGGCTGT | 20 | CAGGTTTGGGAAGATCAGGA | 20 | 139 |

[a] Genes selected based on annotation to RAMs or ability to directly affect Akt or B-cell specific survival signals (see section 3.3.2)

[b] Gene symbols written in **bold** are genes annotated to RAMs. All other genes directly affect Akt or B-cell specific survival signals (see section 3.3.2)

[c] Amplicon size listed in number of nucleotide base pairs (bp)

Table S.2: **RAMs to which genes annotate**

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| ↓ | | ↓ | CF/U | ↓ | ↓ | CF | 1Bii | 313 | 5 | | 1110008J03Rik | Uncharacterized C12orf52 homolog | NM_029096 |
| | | ↓ | U | ↓ | ↓ | CF | 1Bii | 314 | 5 | | 1110008J03Rik | Uncharacterized C12orf52 homolog | NM_029096 |
| | ✖ | ↓ | U | ↓ | ↓ | CF | 1Bii | 315 | 5 | | 1110008J03Rik | Uncharacterized C12orf52 homolog | NM_029096 |
| ↑ | | | | | | | 1Aiii | 263 | 7 | | 1600016N20Rik | Unknown | NM_028050 |
| | | | | | ✖ | U | 1Bi | 224 | 11 | | 1810049H13Rik | Unknown | NM_025560 |
| | | | | | ✖ | U | 1Bii | 163 | 15 | | Acvr1b | Binds ligand, forming receptor complex with 4 transmembrane serine/threonine kinases | NM_007395 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 11 | L1Md_F2 | Acyp2 | Acylphosphatase | NM_029344 |
| | | | | | ↓ | U | 1Aiii | 273 | 7 | | Adamts17 | Glycoprotein, which plays roles in cell signaling, cell fusion, and cell-cell interactions | NM_001033877 |
| ✖ | | | | | | | 1Cii | 296 | 1 | | AK006595 | Unknown | NM_001024489 |
| | | ↓ | U | ↓ | ↓ | CF | 1Aiii | 186 | 14 | | AK019031 | Unknown | |
| ✖ | | | | | | | 1Cii | 296 | 1 | | AK076925 | Unknown | XM_001478622 |
| | ↑ | | | | | | 1Bii | 376 | 5 | RLTR10A B2_Mm2 | Anapc7 | Component of the anaphase promoting complex/cyclosome (APC/C), controling progression through the cell cycle | NM_019805 |
| | | | | | ✖ | CF/U | 1Aiii | 237 | 11 | | Ap1b1 | Subunit of clathrin-associated adaptor protein complex 1 that plays a role in protein sorting | NM_007454 |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 1Bi | 230 | 7 | | Ap2a1 | Clathrin-associated adaptor protein complex 2 subunit that plays a role in protein sorting | NM_001077264 |
| | | ↓ | U | | ↓ | U | 1Cii | 308 | 13 | | Arl10 | ADP-ribosylation factor-like membrane-associated protein | NM_019968 |
| | | | | | ✖ | U | 1Cii | 224 | 11 | | Arl16 | ADP-ribosylation factor-like 16 | NM_197995 |
| | | | | | ↑ | U | 1Aiii | 363 | 16 | | Arvcf | Armadillo repeat gene deleted in velo-cardio-facial syndrome | NM_033474 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[i] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | | ↓ | U | ↓ | ↓ | CF | 1Aiii | 315 | 6 | 1D_B1 | **Atg7** | **Functions as an E1 enzyme essential for multisubstrates (essential for autophagy)** | NM_028835 |
| | ↑ | | | | | | 1Aii | 377 | 3 | B3 | **Bank1** | **B-cell scaffold protein** | NM_001033350 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | **BC016222** | **Predicted Gene** | |
| | ✖ | | | | | | 1Cii | 280 | 7 | | **Bcl7c** | **B-cell CLL/lymphoma 7 protein family member C** | NM_009746 |
| | | | | | ↑ | U | 1Aiii | 262 | 10 | | **Btbd11** | **Ankyrin repeat and BTB/POZ domain-containing protein BTBD11** | NM_028709 |
| | | | | | ✖ | U | 1Ci | 190 | 6 | | **Cadps2** | **$Ca^{2+}$-dependent activator for secretion** | NM_153163 |
| | | | | | ✖ | U | 1Aiii | 224 | 11 | | **Ccdc137** | **Coiled-coil domain-containing protein 137** | NM_152807 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 2 | L1Md_F2 | **Ccdc148** | **Unknown** | NM_001001178 |
| | | | | ✖ | | | 1Cii | 355 | 2 | L1MEc | **Cdh4** | **Cadherin-4 precursor** | NM_009867 |
| ↓ | | | | ↓ | | | 1Cii | 369 | 9 | | **Cldn18** | **Has $Ca^{2+}$-independent cell-adhesion activity involved in intercellular space obliteration** | NM_019815 |
| ↓ | ↓ | ↓ | CF/U | ↓ | ↓ | CF | 1Aiii | 258 | 16 | | **Clic6** | **Probable chloride ion channel.** | NM_172469 |
| ↓ | | | | ↓ | ↓ | U | 1Aiii | 212 | 9 | | **Clstn2** | **May modulate calcium-dependent postsynaptic signals** | NM_022319 |
| | | ↓ | U | | ↓ | U | 1Cii | 308 | 13 | | **Cltb** | **Clathrin light chain B, integral protein in polyhedral coat of coated pits and vesicles** | NM_028870 |
| | | | | | ↑ | U | 1Aii | 444 | X | (TTTG)n | **Cnga2** | **Cyclic nucleotide-gated olfactory channel** | NM_007724 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Cii | 187 | X | L1Md_F2 | **Col4a5** | **Collagen, type IV, alpha 5** | NM_007736 |
| | ↑ | | | | | | 1Aii | 605 | 9 | MIRb | **Crabp1** | **May regulate the access of retinoic acid to nuclear retinoic acid receptors** | NM_013496 |
| | | | | | ✖ | U | 3B | 193 | 5 | L1_Mus3 | **D5Ertd577e** | **Unknown** | NM_177187 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| ↓ | | ↓ | CF/U | ↓ | ↓ | CF | 1Bii | 313 | 5 | | Ddx54 | Implicated in cellular processes involving alteration of RNA secondary structure | NM_028041 |
| | | ↓ | U | ↓ | ↓ | CF | 1Bii | 314 | 5 | | Ddx54 | Implicated in cellular processes involving alteration of RNA secondary structure | NM_028041 |
| | ✖ | ↓ | U | ↓ | ↓ | CF | 1Bii | 315 | 5 | | Ddx54 | Implicated in cellular processes involving alteration of RNA secondary structure | NM_028041 |
| | ✖ | | | | | | 1Bii | 314 | X | | DXBay18 | Unknown | NM_001025384 |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 1Aii | 329 | 7 | | E2F8 | Along with E2F7, inhibitor of E2F-dependent transcription. | NM_001013368 |
| | | | | | ↑ | U | 3C 1Cii | 304 | 19 | | Ehbp1l1 | EH domain-binding protein 1-like protein 1 | NM_053252 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 15 | L1Md_F2 | Fbxl7 | Recognizes phosphorylated proteins and promotes their ubiquitination and degradation | NM_176959 |
| | | | | ↓ | ↓ | CF | 1Aiii | 276 | 9 | MER89 | FOlr4 | Likely folate receptor highly expressed in thymus and spleen | NM_176807 |
| | | | | | ↑ | U | 1Bii | 298 | 18 | | Gjd4 | which materials of low MW diffuse between cells | NM_153086 |
| | ✖ | | | | ✖ | CF | 1Bii | 209 | 1 | | Glul | Glutamine synthetase | NM_008131 |
| ↓ | ↓ | ↓ | CF | ↓ | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | Gm10406 | Predicted Gene | NM_001164727 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm10666 | Predicted Gene | XM_001479387 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm10666 | Predicted Gene | XM_001479387 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | Gm10666 | Predicted Gene | XM_001479387 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | Gm10666 | Predicted Gene | XM_001479387 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | Gm1446 | Predicted Gene | XM_357796 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm1446 | Predicted Gene | XM_357796 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm1446 | Predicted Gene | XM_357796 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | Gm1446 | Predicted Gene | XM_357796 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | Gm1446 | Predicted Gene | XM_357796 |
| | | | | | ✖ | U | 3B | 193 | 5 | L1_Mus3 | Gm16427 | Unknown | XM_001475748 |
| | | | | | ✖ | U | 3B | 193 | 5 | L1_Mus3 | Gm16427 | Unknown | XM_001475748 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | Gm16442 | Predicted Gene | XM_001479154 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm16442 | Predicted Gene | XM_001479154 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm16442 | Predicted Gene | XM_001479154 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | Gm16442 | Predicted Gene | XM_001479154 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | Gm16442 | Predicted Gene | XM_001479154 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | Gm16451 | Predicted Gene | XM_001479381 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm16451 | Predicted Gene | XM_001479381 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm16451 | Predicted Gene | XM_001479381 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | Gm16451 | Predicted Gene | XM_001479381 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | Gm16451 | Predicted Gene | XM_001479381 |
| | | | | | ✖ | U | 3B | 193 | 5 | L1_Mus3 | Gm3139 | Unknown | XM_001476240 |
| | | | | | ✖ | U | 3B | 193 | 5 | L1_Mus3 | Gm3147 | Unknown | XM_001476310 |
| | | | | | ✖ | CF | 3C 1Bii | 150 | 14 | L1Md_F3 | Gm3344 | Predicted Gene | XR_032958 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | Gm3383 | Predicted Gene | XR_032977 |
| | | | | | ✖ | CF | 3C 1Bii | 150 | 14 | L1Md_F3 | Gm3526 | Predicted Gene | XR_033173 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | Gm3629 | Predicted Gene | XR_033294 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | Gm3696 | Predicted Gene | |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | Gm4498 | Predicted Gene | XM_001480490 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm4498 | Predicted Gene | XM_001480490 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm4498 | Predicted Gene | XM_001480490 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | **Gm4498** | **Predicted Gene** | XM_001480490 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | **Gm4498** | **Predicted Gene** | XM_001480490 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 442 | 7 | RLTR10 | **Gm4526** | **Predicted Gene** | XR_034750 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 444 | 7 | RLTR10 | **Gm4526** | **Predicted Gene** | XR_034750 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 444 | 7 | RLTR10 | **Gm4526** | **Predicted Gene** | XR_034750 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 445 | 7 | RLTR10 | **Gm4526** | **Predicted Gene** | XR_034750 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Bii | 445 | 7 | RLTR10 | **Gm4526** | **Predicted Gene** | XR_034750 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 442 | 7 | RLTR10 | **Gm4557** | **Predicted Gene** | XM_001480641 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 444 | 7 | RLTR10 | **Gm4557** | **Predicted Gene** | XM_001480641 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 444 | 7 | RLTR10 | **Gm4557** | **Predicted Gene** | XM_001480641 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 445 | 7 | RLTR10 | **Gm4557** | **Predicted Gene** | XM_001480641 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Bii | 445 | 7 | RLTR10 | **Gm4557** | **Predicted Gene** | XM_001480641 |
| | ✖ | | | | | | 1Bii | 314 | X | | **Gm5640** | **Predicted Gene** | NM_001099302 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | **Gm5794** | **Predicted Gene** | NM_001024712 |
| | | | | | ✖ | CF | 3C 1Aiii | 150 | 14 | L1Md_F3 | **Gm5795** | **Predicted Gene** | XM_619179 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 442 | 7 | RLTR10 | **Gm5890** | **Predicted Gene** | XM_620430 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 444 | 7 | RLTR10 | **Gm5890** | **Predicted Gene** | XM_620430 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Bii | 444 | 7 | RLTR10 | **Gm5890** | **Predicted Gene** | XM_620430 |
| | ↑ | | | | ↑ | U | 3C 1Bii | 445 | 7 | RLTR10 | **Gm5890** | **Predicted Gene** | XM_620430 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Bii | 445 | 7 | RLTR10 | **Gm5890** | **Predicted Gene** | XM_620430 |
| | ✖ | | | | | | 1Cii | 314 | X | | **Gm5936** | **Predicted Gene** | NM_001081670 |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | **Gm6178** | **Predicted Gene** | XM_885146 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | **Gm6178** | **Predicted Gene** | XM_885146 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | **Gm6178** | **Predicted Gene** | XM_885146 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | **Gm6178** | **Predicted Gene** | XM_885146 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | **Gm6178** | **Predicted Gene** | XM_885146 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| ↓ | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 442 | 7 | RLTR10 | Gm8549 | Predicted Gene | XM_989470 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 444 | 7 | RLTR10 | Gm8549 | Predicted Gene | XM_989470 |
| | ↓ | ↓ | CF | ↓ | ↓ | CF | 3C 1Aiii | 444 | 7 | RLTR10 | Gm8549 | Predicted Gene | XM_989470 |
| | ↑ | | | | ↑ | U | 3C 1Aiii | 445 | 7 | RLTR10 | Gm8549 | Predicted Gene | XM_989470 |
| | ↓ | ↓ | CF | ↓ | | | 3C 1Aiii | 445 | 7 | RLTR10 | Gm8549 | Predicted Gene | XM_989470 |
| | | | | ↑ | | | 1Bii | 242 | 9 | | Grm2 | Receptor for glutamate expressed mostly in brain | NM_001160353 |
| | | | | | ✖ | U | 1Bii | 224 | 11 | | Hgs | Involved in intracellular signal transduction. May be a direct effector of $PI_3K$ | NM_008244 |
| | | ↓ | U | | ↓ | U | 1Aii | 308 | 13 | | Higd2a | HIG1 domain family member 2A | NM_025933 |
| | | | | | ✖ | U | 1Aiii | 165 | 14 | | Il17rd | Regulates nuclear ERK signaling, JNK activation, and may be involved in apoptosis | NM_134437 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | X | L1Md_F3 | Il1rapl1 | X-linked interleukin-1 receptor accessory protein-like 1 | NM_001160403 |
| | | | | ↓ | ↓ | CF | 1Aiii | 278 | 14 | | Il3ra | High-affinity IL3 receptor | NM_008369 |
| | | | | | ↑ | U | 1Aiii | 444 | 6 | B1_Mus1 B1_Mm B1F | Jarid1a | Demethylates K4 of histone H3. Regulates Hox protein transcription during differentiation | NM_145997 |
| | | | | | ↑ | U | 3C 1Bii | 304 | 19 | | Kcnk7 | Probable potassium channel subunit | NM_010609 |
| ↑ | | | | | | | 1Ai | 261 | 10 | | Krr1 | Small ribosomal subunit processome component | NM_178610 |
| ↓ | | ↓ | U | ↓ | | | 1Ai | 261 | 10 | | Krr1 | Small ribosomal subunit processome component | NM_178610 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 8 | L1Md_F2 | Large | Glycosyltransferase which participates in glycosylation of alpha-dystroglycan | NM_010687 |
| ↓ | | | | ↓ | | | 1Aiii | 370 | 15 | (CAAAA)n | Lima1 | Inhibits depolymerization and branched filament formation of actin | NM_001113545 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | | ↓ | U | ↓ | ↓ | U | 1Aiii | 215 | 4 | Lx3C | Lingo2 | Leu-rich repeat and Ig-like domain-containing nogo receptor-interacting protein 2 | NM_175516 |
| | | ↓ | U | | | | 1Aiii | 198 | 9 | | Lipc | Catalyzes phospholipid, glyceride, and acyl-CoA thioester hydrolysis | NM_008280 |
| ↑ | | | | | | | 1Bii | 263 | 7 | | Lrrc56 | Leucine-rich repeat-containing protein 56 | NM_153777 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 7 | L1Md_F2 | Luzp2 | Leucine zipper protein 2 | NM_178705 |
| | | | | | ↑ | U | 3C 1Aii | 304 | 19 | | Map3k11 | Activates the JUN N-terminal pathway. Influences microtubule organization | NM_022012 |
| | ✖ | | | | ✖ | CF | 1Aiii | 209 | 1 | | Me9 | Epididymal protein e9-2 | NM_178243 |
| ↓ | | | | ↓ | ↓ | U | 1Aiii | 551 | 19 | MLT1A0 (CATA)n | Myof | Involved in plasmalemma repair and endocytic recycling. Implicated in VEGF signaling | NM_001099634 |
| | | ↓ | U | | ↓ | U | 1Bi | 308 | 13 | | Nop16 | Nucleolar protein 16 | NM_178605 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Ci | 187 | 2 | L1Md_F3 | Nostrin | Multivalent adapter protein which may decrease NOS3 activity | NM_181547 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Cii | 187 | 13 | L1Md_F2 | Npt1 | May be involved in actively transporting phosphate into cells via Na+ cotransport | NM_009198 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Cii | 187 | 13 | L1Md_F2 | Npt4 | May be involved in actively transporting phosphate into cells via Na+ cotransport | NM_134069 |
| ↓ | | ↓ | CF | | ↓ | CF/U | 1Aiii | 309 | 13 | | Ntrk2 | Tyrosine-protein kinase receptor involved in nervous system development, maintenance | NM_001025074 |
| | | | | | ↑ | U | 3C 1Bii | 304 | 19 | | Pcnxl3 | Pecanex-like protein 3 | NM_144868 |
| | | | | | ✖ | U | 1Bii | 224 | 11 | | Pde6g | cGMP-PDE likely involved in G-protein-mediated phototransduction in vertebrates | NM_012065 |
| ↓ | | | | ↓ | ↓ | CF | 1Aiii | 272 | 15 | | Pdzd2 | Unknown | NM_008389 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[J] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 1Bi | 230 | 4 | | Pgm2 | Participates in CF/U the breakdown and synthesis of glucose | NM_028132 |
| | | ↓ | U | | | | 1Aiii | 202 | 8 | | Pkd1l2 | May function as an ion-channel regulator or G-protein-coupled receptor | NM_029686 |
| | | ↓ | U | | | | 1Cii | 198 | 15 | | Poldip3 | Polymerase delta-interacting protein 3 | NM_178627 |
| | | | | | ↑ | U | 1Aiii | 306 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| | | | | | ↑ | U | 1Aiii | 308 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| ↓ | | ↓ | CF/U | ↓ | ↓ | CF/U | 1Aiii | 311 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| | ✖ | | | | | | 1Aiii | 311 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| ↓ | ✖ | ↓ | CF/U | ↓ | ↓ | CF | 1Aiii | 312 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| ↓ | ✖ | ↓ | CF/U | ↓ | ↓ | CF | 1Aiii | 313 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| | | ↓ | U | ↓ | ↓ | CF | 1Aiii | 314 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| | ✖ | | | | | | 1Aiii | 315 | 6 | | Prickle2 | Prickle-like 2 protein likely involved in Wnt signaling | NM_001081146 |
| ↑ | | | | | ↑ | U | 1Bi | 262 | 1 | | Qsox1 | Quiescin Q6 sulfhydryl oxidase 1 | NM_001024945 |
| | ↑ | | | | | | 1Aiii | 171 | 2 | MIRc | Ralgds | Stimulates dissociation of GDP from Ras-related GTPases | NM_009058 |
| ↑ | | | | | | | 1Cii | 263 | 7 | | Rassf7 | Ras association domain-containing protein 7 | NM_025886 |
| | | ↓ | U | | | | 1Aii | 198 | 15 | | Rrp7a | Ribosomal RNA-processing protein 7 homolog A | NM_029101 |
| | | ↓ | U | | | | 1Aiii | 198 | 15 | | Serhl | Probable serine hydrolase | NM_023475 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | | | | ✖ | | | 1Aiii | 209 | 1 | | Sgpp2 | High phosphohydrolase activity against dihydrosphingosine-1-phosphate and S1P | NM_001004173 |
| ↓ | | ↓ | U | ↓ | | | 1Aii | 262 | 17 | | Six3 | Involved in visual development | NM_011381 |
| ↑ | | | | | ↑ | U | 1Aii | 262 | 17 | | Six3 | Involved in visual development | NM_011381 |
| | | | | | ↑ | U | 1Aiii | 307 | 15 | | Slc11a2 | Important in macrophage-specific membrane transport, specifically of metal ions | NM_008732 |
| | | | | | ↑ | U | 1Aiii | 366 | 13 | | Slc38a9 | Putative sodium-dependent amino acid/proton antiporter | NM_178746 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 1 | L1Md_F2 | Slco6d1 | Unknown | NM_027584 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Cii | 187 | 2 | L1Md_F3 | Spbc25 | Component of the essential NDC80 complex. Required for kinetochore integrity | NM_025565 |
| ✖ | | | | | ✖ | CF | 1Aii | 206 | 2 | | Srms | Non-receptor tyrosine kinase likely involved in keratinocyte proliferation/differentiation | NM_011481 |
| | | | | ✖ | | | 1Cii | 355 | 2 | L1MEc | Taf4a | Part of complex potentiating regulation via various transcriptional activators | NM_001081092 |
| | ✖ | | | | ✖ | U | 1Bii | 166 | 8 | | Taf5l | efficiently acetylating histones in a nucleosomal context | NP_598727 |
| ↓ | | | | ↓ | | | 1Aiii | 193 | 18 | | Tcf4 | Participates in Wnt signaling and modulates MYC transcription | NM_009333 |
| | | ↓ | U | | | | 1Aiii | 200 | 18 | | Tcf4 | Participates in Wnt signaling and modulates MYC transcription | NM_009333 |
| | ✖ | | | | ✖ | CF | 1Cii | 209 | 1 | | Teddm1 | Transmembrane epididymal protein 1 | NM_178244 |
| ✖ | | | | ↑ | ✖ | CF | 1Aiii | 242 | 9 | | Tex264 | Unknown | NM_001081654 |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 1Bii | 329 | 7 | | Th | Important in adrenergic neurons physiology | NM_009377 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[fg] | | | | | | | | |
| ↑ | | | | | | | 1Bi | 260 | 11 | | Tha1 | L-threonine aldolase catalyzing conversion of L-threonine to glycine and acetaldehyde | NM_027919 |
| | ↑ | | | | | | 1Cii | 162 | 10 | | Theg | May be involved (but not essential) in spermatogenesis | NM_011583 |
| | | | | ✖ | | | 1Aiii | 208 | 5 | | Tpst2 | Catalyzes the O-sulfation of tyrosine residues within acidic motifs of polypeptides | NM_009419 |
| ↓ | | ↓ | U | ↓ | ↓ | CF | 1Ci | 186 | 14 | | Ubac2 | Ubiquitin-associated domain-containing protein 2 | NM_026861 |
| | | | | | ↑ | U | 1Bii | 266 | 2 | | Ube2l6 | Ubiquitin attachment catalyst | NM_019949 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 3 | L1Md_F3 | Unc5c | May regulate pro-survival signaling | NM_009472 |
| | | ↓ | U | ↓ | ↓ | CF | 3C 1Aiii | 187 | 3 | L1Md_F2 | Usp13 | Ubiquitin specific peptidase 13 | NM_001013024 |
| | | | | ↓ | ↓ | CF | 1Aiii | 276 | 6 | (CCG)n | Wbp1 | WWP1 and WWP2. Interacts with WWOX and NEDD4 | NM_016757 |
| ✖ | | | | | ✖ | CF | 1Aiii | 101 | 11 | | Wscd1 | Unknown | NM_177618 |
| | | | | | ✖ | U | 1Aiii | 165 | 11 | | Wscd1 | Unknown | NM_177618 |
| | | | | | ✖ | U | 1Aiii | 166 | 11 | | Wscd1 | Unknown | NM_177618 |
| ↓ | | | | ↓ | | | 1Aiii | 358 | 11 | | Wscd1 | Unknown | NM_177618 |
| ↓ | ✖ | ✖ | U | ↓ | | | 1Aiii | 359 | 11 | | Wscd1 | Unknown | NM_177618 |
| | ✖ | | | | | | 1Aiii | 360 | 11 | | Wscd1 | Unknown | NM_177618 |
| | ✖ | | | | | | 1Aiii | 362 | 11 | | Wscd1 | Unknown | NM_177618 |
| | ✖ | | | | ↑ | U | 1Aiii | 366 | 11 | | Wscd1 | Unknown | NM_177618 |
| | ✖ | | | | | | 1Aiii | 314 | X | | Xlr5a | X-linked lymphocyte-regulated 5A | NM_001045539 |
| | ✖ | | | | | | 1Aiii | 314 | X | | Xlr5c | X-linked lymphocyte-regulated 5C | NM_031493 |
| ✖ | | | | ↑ | ✖ | CF/U | 1Bii | 238 | 7 | | Zfp128 | Plays critical role in mediating BMP signaling and interacts with SMAD proteins | NM_153802 |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| ✖ | | | | ↑ | ✖ | CF/U | 1Ai | 238 | 7 | | Zscan22 | Zinc finger and SCAN domain containing 22 | NM_001001447 |
| | | | | | ✖ | U | 2 | 143 | 5 | MIRb | | | |
| | | | | | ✖ | CF | 3C 2 | 150 | 11 | L1Md_F3 | | | |
| ↓ | | ↓ | U | ↓ | ↓ | CF | 4 | 187 | | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 1 | L1Md_F3 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 3 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 4 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 6 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 7 | RLTR10 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 8 | L1Md_F3 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 8 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 13 | L1Md_F3 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 14 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 16 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 18 | L1Md_F2 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 18 | L1Md_F3 | | | |
| | | ↓ | U | ↓ | ↓ | CF | 3C 2 | 187 | 19 | L1Md_F2 | | | |
| | ✖ | | | ✖ | ✖ | CF | 2 | 208 | 8 | | | | |
| ↓ | | ↓ | U | ↓ | ↓ | U | 2 | 222 | 14 | PB1D9 | | | |
| | ↓ | ↓ | U | | ↓ | U | 2 | 223 | 14 | B4A | | | |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 2 | 228 | 7 | B4A | | | |
| ↓ | ↓ | ↓ | CF/U | ↓ | | | 2 | 231 | 9 | RLTR10A | | | |
| | | | | ↑ | | | 2 | 238 | 8 | | | | |
| | | | | ↑ | | | 2 | 239 | 9 | RLTR10A | | | |
| | | | | | ✖ | U | 2 | 240 | X | | | | |

**Table S.2** – continued

| Methylation Status[b] | | | | | | | Class.[h] | RAM bp | Chrom.[j] | Repeat Element | Gene Symbol | Gene Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPS[c] | 3μg/kg TCDD[d] | 3μg/kg TCDD+LPS[e] | | 30μg/kg TCDD[f] | 30μg/kg TCDD+LPS[g] | | | | | | | | |
| | | | | ↑ | | | 2 | 241 | 1 | IAPEY3_LTR | | | |
| ↓ | | | | ↓ | | | 2 | 245 | 9 | | | | |
| ↓ | | | | ↓ | | | 2 | 245 | 14 | | | | |
| ↓ | | | | ↓ | | | 2 | 246 | 13 | | | | |
| ↓ | | ↓ | U | ↓ | ↓ | CF/U | 2 | 269 | 13 | RLTR10A | | | |
| ↓ | | | | ↓ | ↓ | CF/U | 2 | 271 | 3 | | | | |
| ↓ | | | | ↓ | ↓ | CF | 2 | 272 | 3 | | | | |
| | | | | ↓ | | | 2 | 287 | 12 | | | | |
| ↓ | | | | ↓ | | | 2 | 288 | 9 | RLTR1 | | | |
| | | | | ✖ | | | 2 | 294 | 3 | | | | |
| ✖ | | | | ✖ | | | 2 | 296 | 1 | | | | |
| | ↓ | ↓ | U | ↓ | | | 2 | 303 | 14 | L1M4c | | | |
| | | | | | ↑ | U | 2 | 306 | 12 | | | | |
| | | ↓ | U | | | | 2 | 323 | 1 | RMER17A | | | |
| | | ↓ | U | | | | 2 | 324 | 1 | RMER17A | | | |
| | | ↓ | U | | | | 2 | 328 | 5 | ORR1C1-int | | | |
| ↓ | ↓ | ↓ | CF | ↓ | | | 2 | 331 | 1 | RMER17A | | | |
| ↓ | | ↓ | U | ↓ | ↓ | U | 2 | 356 | 15 | L1Md_F2 RLTR10A | | | |
| | ↑ | ✖ | U | ✖ | | | 2 | 356 | 15 | L1Md_F2 RLTR10A | | | |
| ↓ | ✖ | | | ↓ | | | 2 | 370 | 5 | RLTR10A | | | |
| | ↑ | | | | | | 2 | 378 | 14 | PB1D9 | | | |
| | ↑ | | | | | | 2 | 379 | 7 | | | | |
| | ✖ | | | | | | 2 | 560 | 11 | | | | |
| | ✖ | | | | | | 2 | 574 | 2 | | | | |
| | | | | | ↓ | U | 2 | 603 | 14 | PB1D9 | | | |

**Table S.2** – continued

[a]Cloning and subsequent sequencing of arbitrarily primed PCR products annotated 79% (82) of identified RAMs

[b]RAM methylation status is indicated as: a) Hypomethylation ( ↓ -significant decrease in methylation when compared to control); b) hypermethylation (↑ -significant increase in methylation when compared to control); or c) new methylation (×-PCR product formed in treatment that did not form in control). RAMs observed in 3μg/kg TCDD+LPS and 30μg/kg TCDD+LPS treatment are divided into Carry Forward (CF-observed in TCDD and/or LPS treatment alone as well as TCDD+LPS treatment), Unique (U-observed only in TCDD+LPS treatment), or CF/U (observed as CF and U). Annotated genes are identified as CF/U when the sequenced PCR product is within 2bp of carry forward and unique RAM(s) and so it is impossible to discern which RAM it represents (see Materials and Methods PCR Products Annotating to ≥2 RAMs)

[c]Of the 40 RAMs identified in LPS solo treatment, 63% were annotated: 100% (3/3) of hyper-, 59% (19/32) of hypo-, and 80% (4/5) of new methylations

[d]Of the 43 RAMs identified in 3μg/kg TCDD solo treatment, 56% were annotated: 54% (7/13) of hyper-, 58% (7/12) of hypo-, and 56% (10/18) of new methylations

[e]Of the 34 RAMs identified in 3μg/kg TCDD+LPS treatment, 62% were annotated: 69% (20/29) of hypo- and 25% (1/4) of new methylations

[f]Of the 42 RAMs identified in 30μg/kg TCDD solo treatment, 64% were annotated: 100% (1/1) of hyper-, 59% (22/37) of hypo-, and 100% (4/4) of new methylations

[g]Of the 39 RAMs identified in 30μg/kg TCDD+LPS treatment, 78% were annotated: 100% (5/5) of hyper-, 73% (17/23) of hypo-, and 79% (11/14) of new methylations

[h]Classification is listed according to scheme outlined in Figure S.1

[j]Chromosome encoding gene to which RAM annotates. Those RAMs without a chromosome listed annotate to many regions of many chromosomes

[k]Repeat elements: LINE elements (L1M4c, L1Md_F2, L1Md_F3, L1MEc, L1_Mus3, Lx3C), LTR elements (IAPEY3_LTR, MER89, MLT1A0, ORR1C1-int, RLTR1, RLTR10, RLTR10A, RMER17A), SINE elements (B1F, B1_Mus1, B1_Mm, B2_Mm2, B3, 1D_B1, B4A, MIRb, MIRc, PB1D9), and Simple_repeat elements ((CAAA)n, (CATA)n, (CCG)n, (TTG)n)

Table S.3: **Signaling pathways and cellular processes represented by annotated genes**[a]

| LPS | | 3μg/kg | | 3μg/kg TCDD+LPS | | 30μg/kg | | 30μg/kg TCDD+LPS | |
|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** |
| **Apoptosis**[c] | | | | | | | | | |
| | | Bcl7c | NM_009746 | Atg7 | NM_028835 | Atg7 | NM_028835 | Atg7 | NM_028835 |
| | | | | Unc5c | NM_009472 | Unc5c | NM_009472 | Unc5c | NM_009472 |
| **Calcium Ion Storage** | | | | | | | | | |
| Clstn2 | NM_022319 | | | Cltb | NM_028870 | Cdh4 | NM_009867 | Cadps2 | NM_153163 |
| | | | | Pkd1l2 | NM_029686 | Clstn2 | NM_022319 | Clstn2 | NM_022319 |
| | | | | | | | | Cltb | NM_028870 |
| **Cell Cycle**[d] | | | | | | | | | |
| E2F8 | NM_001013368 | Anapc7 | NM_019805 | E2F8 | NM_001013368 | E2F8 | NM_001013368 | Spbc25 | NM_025565 |
| | | E2F8 | NM_001013368 | Spbc25 | NM_025565 | Spbc25 | NM_025565 | | |
| **Cell Differentiation** | | | | | | | | | |
| Ntrk2 | NM_001025074 | Theg | NM_011583 | Ntrk2 | NM_001025074 | | | Ntrk2 | NM_001025074 |
| **Cell Proliferation** | | | | | | | | | |
| E2F8 | NM_001013368 | E2F8 | NM_001013368 | E2F8 | NM_001013368 | E2F8 | NM_001013368 | | |
| **Chromatin Modification** | | | | | | | | | |
| | | | | | | | | Jarid1a | NM_145997 |
| **Innate Immunity**[e] | | | | | | | | | |
| | | Bank1 | NM_001033350 | Il1rapl1 | NM_001160403 | Il1rapl1 | NM_001160403 | Il1rapl1 | NM_001160403 |

**Table S.3** – continued

| LPS | | 3µg/kg | | 3µg/kg TCDD+LPS | | 30µg/kg | | 30µg/kg TCDD+LPS | |
|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** |
| **Ion**[f] | | | | | | | | | |
| Clic6 | NM_172469 | Bank1 | NM_001033350 | Clic6 | NM_172469 | Clic6 | NM_172469 | Cadps2 | NM_153163 |
| Lima1 | NM_001113545 | Clic6 | NM_172469 | Npt1 | NM_009198 | Lima1 | NM_001113545 | Cadps2 | NM_153163 |
| Pgm2 | NM_028132 | Pgm2 | NM_028132 | Npt4 | NM_134069 | Npt1 | NM_009198 | Clic6 | NM_172469 |
| Prickle2 | NM_001081146 | Prickle2 | NM_001081146 | Pgm2 | NM_028132 | Npt4 | NM_134069 | Cnga2 | NM_007724 |
| Th | NM_009377 | Th | NM_009377 | Prickle2 | NM_001081146 | Pgm2 | NM_028132 | Jarid1a | NM_145997 |
| Zfp128 | NM_153802 | | | Th | NM_009377 | Prickle2 | NM_001081146 | Kcnk7 | NM_010609 |
| Zscan22 | NM_001001447 | | | | | Th | NM_009377 | Npt1 | NM_009198 |
| | | | | | | Zfp128 | NM_153802 | Npt4 | NM_134069 |
| | | | | | | Zscan22 | NM_001001447 | Prickle2 | NM_001081146 |
| | | | | | | | | Slc11a2 | NM_008732 |
| | | | | | | | | Slc38a9 | NM_178746 |
| | | | | | | | | Zfp128 | NM_153802 |
| | | | | | | | | Zscan22 | NM_001001447 |
| **Kinase**[g] | | | | | | | | | |
| Ntrk2 | NM_001025074 | Bank1 | NM_001033350 | Ntrk2 | NM_001025074 | | | Acvr1b | NM_007395 |
| Srms | NM_011481 | | | | | | | Map3k11 | NM_022012 |
| | | | | | | | | Ntrk2 | NM_001025074 |
| | | | | | | | | Srms | NM_011481 |

**Table S.3** – continued

| LPS | | 3µg/kg | | 3µg/kg TCDD+LPS | | 30µg/kg | | 30µg/kg TCDD+LPS | |
|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** |
| **Protein Transport**[h] | | | | | | | | | |
| Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap1b1 | NM_007454 |
| | | Crabp1 | NM_013496 | Atg7 | NM_028835 | Atg7 | NM_028835 | Atg7 | NM_028835 |
| | | | | Cltb | NM_028870 | | | Cadps2 | NM_153163 |
| | | | | | | | | Cltb | NM_028870 |
| | | | | | | | | Hgs | NM_008244 |
| **RedOx**[i] | | | | | | | | | |
| Qsox1 | NM_001024945 | Th | NM_009377 | Th | NM_009377 | Th | NM_009377 | Qsox1 | NM_001024945 |
| Th | NM_009377 | | | | | | | Jarid1a | NM_145997 |
| **Transcription**[j] | | | | | | | | | |
| Ddx54 | NM_028041 | Ddx54 | NM_028041 | Ddx54 | NM_028041 | Ddx54 | NM_028041 | Ddx54 | NM_028041 |
| E2F8 | NM_001013368 | E2F8 | NM_001013368 | E2F8 | NM_001013368 | E2F8 | NM_001013368 | Jarid1a | NM_145997 |
| Six3 | NM_011381 | Taf5l | NP_598727 | Nostrin | NM_181547 | Nostrin | NM_181547 | Nostrin | NM_181547 |
| Tcf4 | NM_009333 | | | Six3 | NM_011381 | Six3 | NM_011381 | Six3 | NM_011381 |
| Zfp128 | NM_153802 | | | Tcf4 | NM_009333 | Taf4a | NM_001081092 | Taf5l | NP_598727 |
| Zscan22 | NM_001001447 | | | | | Tcf4 | NM_009333 | Zfp128 | NM_153802 |
| | | | | | | Zfp128 | NM_153802 | Zscan22 | NM_001001447 |
| | | | | | | Zscan22 | NM_001001447 | | |
| **Ubiquitin**[k] | | | | | | | | | |
| | | | | Atg7 | NM_028835 | Atg7 | NM_028835 | Atg7 | NM_028835 |
| | | | | Usp13 | NM_001013024 | | | Ube2l6 | NM_019949 |

**Table S.3** – continued

| LPS | | 3μg/kg | | 3μg/kg TCDD+LPS | | 30μg/kg | | 30μg/kg TCDD+LPS | |
|---|---|---|---|---|---|---|---|---|---|
| **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** | **Gene** | **NCBI RefSeq** |
| **Vesicles**[l] | | | | | | | | | |
| Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap2a1 | NM_001077264 | Ap1b1 | NM_007454 |
| | | | | Cltb | NM_028870 | Nostrin | NM_181547 | Cadps2 | NM_153163 |
| | | | | Nostrin | NM_181547 | | | Cltb | NM_028870 |
| | | | | | | | | Nostrin | NM_181547 |

[a] Signaling pathway and cellular process involvement discerned via GO (Gene Ontology) analysis, genes are grouped based on (single or many related) GO terms.

[b] Treatment groups are marked as having at least one representative annotated gene (✓) or none (–)

[c] Genes involved in (positive and negative) regulation of apoptosis

[d] Genes involved in the cell cycle and mitosis

[e] Genes involved in B-cell activation and innate immune response

[f] Genes involved in calcium ion homeostasis, ion transport, and metal ion binding

[g] Genes involved in protein kinase binding and kinase activity

[h] Genes involved in protein transport, intracellular protein transport, and protein transporter activity

[i] Genes involved in oxidation reduction and oxidoreductase activity

[j] Genes involved in transcription, transcription repressor activity, and transcription from RNA polymerase II promoter

[k] Genes involved in ubiquitin thiolesterase activity, regulation of protein ubiquitination, and ubiquitin-protein ligase activity

[l] Genes involved in endocytosis, exocytosis, and vesicle-mediated transport

Table S.4 : **Fold change in mRNA expression of 14 selected[a] genes as measured using qRT-PCR**

[a]Genes selected based on annotation to genes or ability to directly affect Akt and/or B-cell specific survival signals (see section 3.3.2

[b]Gene symbols written in bold are genes annotated to RAMs. All others are genes which directly affect Akt and/or B cell specific survival signals

[c]All fold changes (based on qRT-PCR analysis) are statistically significant (as measured by Student's, two-tailed, t-test, $p \leq 0.05$) with direction and extent of disregulation listed: upregulation (↑), downregulation (↓), or no change (–)

[d]Out of 6 animals per treatment group, the number with mRNA expression outside of the control 95% confidence interval (CI) are listed: above 95% CI (↑), below 95% CI (↓), none (–)

| Gene Symbol[b] | LPS | | | | 30μg/kg TCDD | | | | 30μg/kg TCDD+LPS | | | | Description/Function | NCBI RefSeq |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fold Change[c] | | Individual Data[d] | | Fold Change[c] | | Individual Data[d] | | Fold Change[c] | | Individual Data[d] | | | |
| Adcy5 | ↑ | 4.85 | ↑ | 4 | ↓ | 0.23 | ↑ | – | ↓ | 0.42 | ↑ | – | Ca++ dependent Adenylcyclase | NM_001012765 |
| | | | ↓ | – | | | ↓ | 5 | | | ↓ | 6 | | |
| Akt | ↑ | 2.38 | ↑ | 5 | – | 5.71 | ↑ | 2 | – | 1.35 | ↑ | 3 | Serine/Threonine Kinase Capable of regulating proliferation | NM_009652 |
| | | | ↓ | – | | | ↓ | 1 | | | ↓ | 1 | | |
| Bcor | ↓ | 0.80 | ↑ | – | – | 0.80 | ↑ | 2 | – | 1.03 | ↑ | 1 | Bcl6 Co-Repressor & transcriptional regulator May Effect Epigenetic Modifications | NM_175045 |
| | | | ↓ | 5 | | | ↓ | 2 | | | ↓ | 2 | | |
| **Zfp128** | ↓ | 0.61 | ↑ | – | – | 0.84 | ↑ | 1 | – | 0.96 | ↑ | 1 | Represses BMP/TGF-β signaling through SMAD1 | NM_153802 |
| | | | ↓ | 5 | | | ↓ | 3 | | | ↓ | 2 | | |
| **Ddx54** | – | 0.80 | ↑ | – | ↓ | 0.16 | ↑ | – | ↓ | 0.04 | ↑ | – | RNA Helicase able to repress nuclear receptor transcriptional activity | NM_028041 |
| | | | ↓ | 1 | | | ↓ | 5 | | | ↓ | 6 | | |
| Phlpp | – | 1.21 | ↑ | 3 | ↓ | 0.13 | ↑ | – | ↓ | 0.12 | ↑ | – | Inhibitor of Akt phosphorylation and activity | NM_133821 |
| | | | ↓ | – | | | ↓ | 4 | | | ↓ | 5 | | |
| Ube2l6 | – | 0.89 | ↑ | – | ↓ | 0.25 | ↑ | – | ↓ | 0.12 | ↑ | – | Ubiquitin-Conjugating Enzyme | NM_019949 |
| | | | ↓ | 3 | | | ↓ | 5 | | | ↓ | 6 | | |
| **Ralgds** | – | 0.77 | ↑ | – | ↓ | 0.20 | ↑ | – | ↓ | 0.05 | ↑ | – | Stimulates dissociation of GDP from Ras-related GTPases | NM_009058 |
| | | | ↓ | 3 | | | ↓ | 5 | | | ↓ | 6 | | |
| **Bank1** | – | 1.58 | ↑ | 3 | ↓ | 0.13 | ↑ | – | ↓ | 0.25 | ↑ | – | B-Cell adaptor protein capable of inhibiting Akt activation | NM_001033350 |
| | | | ↓ | – | | | ↓ | 4 | | | ↓ | 5 | | |
| **IL17rd** | ↓ | 0.60 | ↑ | – | ↓ | 0.56 | ↑ | – | ↓ | 0.55 | ↑ | – | Inhibits FGF signaling (and so proliferation) through the FGFR | NM_134437 |
| | | | ↓ | 5 | | | ↓ | 4 | | | ↓ | 6 | | |
| Ptpn3 | ↓ | 0.70 | ↑ | – | – | 0.89 | ↑ | 1 | ↓ | 0.71 | ↑ | – | Protein Tyrosine Phosphatase, Non-Receptor Type 3 | NM_011207 |
| | | | ↓ | 5 | | | ↓ | 2 | | | ↓ | 5 | | |
| **Cadps2** | – | 0.70 | ↑ | 1 | – | 0.88 | ↑ | 1 | – | 0.77 | ↑ | 1 | Ca++ dependent secretion activator | NM_153163 |
| | | | ↓ | 4 | | | ↓ | 2 | | | ↓ | 4 | | |
| **Krr1** | – | 0.86 | ↑ | 1 | – | 0.89 | ↑ | 2 | – | 1.03 | ↑ | 1 | Small ribosomal subunit processome component | NM_178610 |
| | | | ↓ | 5 | | | ↓ | 3 | | | ↓ | 2 | | |
| RARα | – | 0.92 | ↑ | – | – | 1.29 | ↑ | 3 | – | 1.31 | ↑ | 4 | Retinoic acid receptor controling cell function by directly regulating gene expression | NM_009024 |
| | | | ↓ | 1 | | | ↓ | – | | | ↓ | – | | |

**Table S.5: Literature references for annotated genes.** The numbered references correspond to the numbers above lines indicating interactions in figures S2-4.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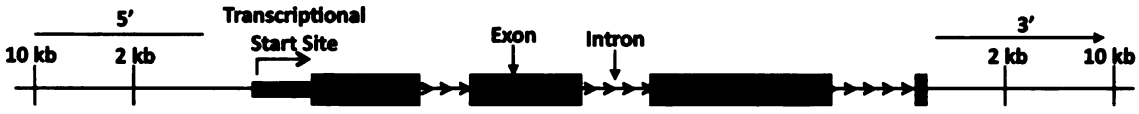
## Appendix B: Supplemental Figures




1) Associated with a gene
   - A. Within a gene
     - i. Spans transcriptional start site and/or 5' untranslated region
     - ii. Exon
     - iii. Intron
   - B. Upstream from transcriptional start site
     - i. ≤2kb
     - ii. ≤10kb and >2kb
   - C. Downstream from last exon/intron
     - i. ≤2kb
     - ii. ≤10kb and >2kb
2) >10kb from either transcriptional start site or last exon/intron
3) Repeat element: multiple "top" hit scores and one/several are associated with gene(s)
   - A. One site and one gene
   - B. Multiple sites in one gene
   - C. Two or more genes
4) Repeat element: multiple "top" hit scores and none associated with gene(s)

Figure S.1.: **Classification of RAM annotation based upon genomic location, as determined by BLAT search.** Our scheme used to classify annotated Regions of Altered Methylation (RAMs) according to information gleaned from BLAST-like Sequence Alignment Tool (BLAT; http://genome.ucsc.edu/cgi-bin/hgBlat) analysis based on where, in relation to a gene, the PCR product aligned (i.e., the RAM was identified). For example, PCR products designated as 1Bii are located between 2 and 10kb upstream from an annotated gene.

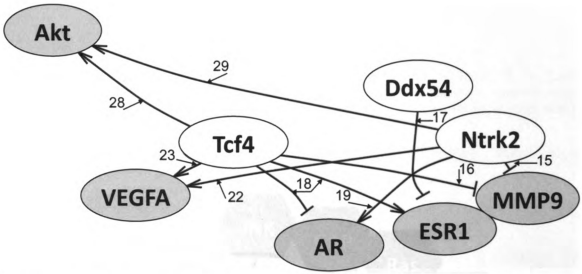



Figure S.2.: **Regulatory interactions of annotated genes in 25$\mu$g LPS treatment.** Replicate of Figure 2.3A with references designated as numbers above lines indicating regulation. Specific references corresponding to these numbers are listed in Table S.5.

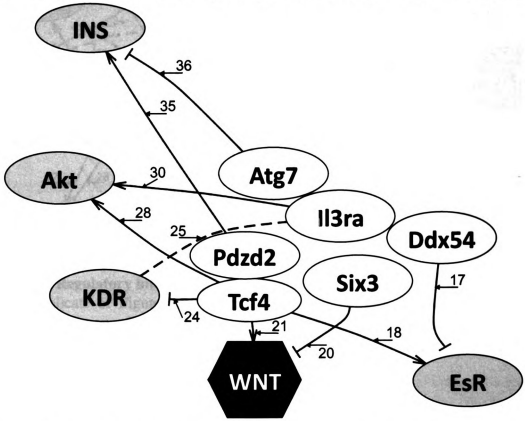



Figure S.3.: **Regulatory interactions of annotated genes in 30$\mu$g/kg TCDD treatment.** Replicate of Figure 2.3B with references designated as numbers above lines indicating regulation. Specific references corresponding to these numbers are listed in Table S.5.

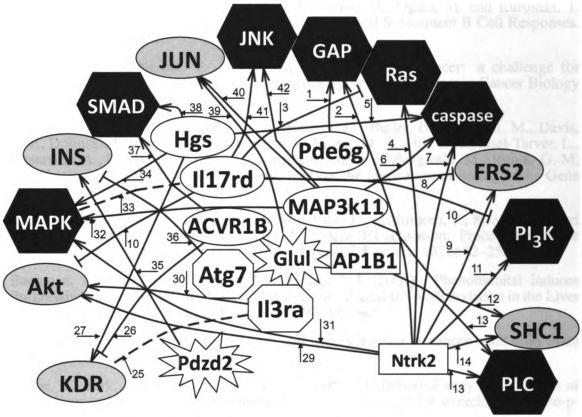



Figure S.4.: **Regulatory interactions of annotated genes in 30$\mu$g/kg TCDD+LPS treatment.** Replicate of Figure 2.3C with references designated as numbers above lines indicating regulation. Specific references corresponding to these numbers are listed in Table S.5.

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